Research Article

Preparation of an Inactivated Polyvalent Vaccine Against Common Bacterial Pathogens Causing Bovine Mastitis and Evaluation of its' Immunizing and Therapeutic Potentials

Rafik Soliman[1*], Rafik H. Sayed[2], Hanan Mahmoud[3], Heidy Abo El-Yazeed[1], Marsell Saad[1], Shaimaa Abdelall Elsaady[2], Khalid Sh[3]

[1]Department of Microbiology and Immunology, Faculty of Veterinary Medicine, Cairo University, Egypt; [2]Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Agricultural Research Center (ARC), Ministry of Agriculture, Abbasia, Cairo, Egypt; [3]Animal Health Research Institute (AHRI), Agricultural Research Center (ARC), Ministry of Agriculture, Giza, Egypt.

Abstract | A total of 3608 quarter's milk samples were aseptically collected form 902 Friesian cows, of which 68 (7.54%) suffered from clinical mastitis and 120 (13.3%) proved to suffer from subclinical form of mastitis. Bacteriological examination of the milk samples revealed the isolation of the following pathogens from clinical and subclinical mastitis cases, respectively; *E. coli* (29.41% and 29.2%), *S. aureus* (19.12% and 25%), *Str. agalactiae* (16.2% and 16.6%), *Str. dysgalactiae* (14.7% and 20%), *Str. uberis* (7.3% and 2.5%), *K. pneumoniae* (4.4% and 3.3%), *Str. fecalis* (4.4% and 0%), *Str. pyogenes* (2.9% and 0%) and *Pseudomonas aeruginosa* (1.5% and 3.3%). Among the recovered *E. coli* serotypes, the serotype O111:K58 (B4) was the most prevalent (38.2%). An inactivated polyvalent autogenous mastitis vaccine was prepared from the most encountered bacterial pathogens, namely, *E. coli*, *S. aureus*, *Str. agalactiae and Str. dysgalactiae*. The selected strains for vaccine preparation were inactivated with a predetermined minimal lethal dose of gamma radiation (4krad kGy/min) and the bacterial inactivation was assured using bacteriological examination. Two experiments were done; in experiment No.1, the immunizing efficacy of the prepared vaccine was evaluated in two groups of apparently normal lactating cows (4 cows /group). The first group was unvaccinated and kept as a control group all over the study while the animals in the second group were immunized with the prepared vaccine. Vaccinated cows received two vaccinal doses (5ml) at two week interval. The vaccine was injected subcutaneously in the brachiocephalic muscle. In experiment No. 2, the therapeutic potential of the prepared vaccine was tested in four groups of lactating cows suffering from subclinical mastitis. The first group was left untreated. The second group was treated only with antibiotics (neomycin and penicillin) and the third group was immunized with the prepared vaccine alone. The fourth group was treated simultaneously both with the antibiotics and the prepared vaccine. In both experiments blood and milk samples from cows in all groups were collected before and at 2 weeks intervals post immunization and/or antibiotic treatment. Vaccinated cows in the two experiments did not develop clinical mastitis during the whole observation period that extends up to 20 weeks post treatment. The total immunoglobulin concentration (g/dl) measured four weeks post immunization was significantly higher in the serum and milk whey of immunized cows as compared to the non-immunized cows ($P<0.01$). The highest ELISA titers against *E. coli* (O111), *S. aureus, Str. agalactiae* and *Str. dysgalactiae* were recorded 4 weeks post immunization in the serum and milk whey of immunized cows as compared to the non-immunized group. In cows suffering from subclinical mastitis the vaccination and /or antibiotic treatment induced significant decrease in the somatic cell counts ($P<0.01$). Also, significant reduction of the CFU/ml of the examined milk samples ($P<0.01$) and spontaneous recovery were recorded. No case proceeds to clinical mastitis for an observation period of 20 weeks. These finding showed that the prepared vaccine is potent and able to protect cow against mastitis and able to reduce the disease severity in infected cows.

Keywords | Bovine mastitis, bacterial pathogens, polyvalent inactivated vaccine, Somatic cell count (SCC), immunoglobulins level, ELISA antibody titer

Received | August 19 2022; **Accepted** | September 20, 2022; **Published** | April 10, 2023
***Correspondence** | Rafik Soliman, Department of Microbiology and Immunology, Faculty of Veterinary Medicine, Cairo University, Egypt; **Email:** rafiksoliman108@cu.edu.eg
Citation | Soliman R, Sayed RH, Mahmoud H, El-Yazeed HA, Saad M, Elsaady SA, Sh K (2023). Preparation of an inactivated polyvalent vaccine against common bacterial pathogens causing bovine mastitis and evaluation of its' immunizing and therapeutic potentials. J. Anim. Health Prod. 11(2): 99-108.
DOI | http://dx.doi.org/10.17582/journal.jahp/2023/11.2.99.108
ISSN | 2308-2801

INTRODUCTION

Bovine mastitis is an economically important disease that affects the dairy farms and the national budget in two ways; direct cost due to per-acute forms of mastitis and loss of cows through premature culling and indirect costs due to reduced milk production and quality, expensive antibiotic therapy, and the serious side effects of the used antibiotics. Excessive use of antibiotics may lead to complete removal of all bacteria, the pathogenic and non-pathogenic, an effect, which has been reported to be associated with a marked increase in severity of acute mastitis (Khaitsa et al., 2000, Schlegelov à et al., 2002);

Mastitis can be caused by a wide range of organisms, including gram-negative and gram-positive bacteria, mycoplasmas, and algae. Many microbial species that are common causes of bovine mastitis, such as *E. coli*, *Klebsiella pneumoniae, Str. agalactiae* and *S. aureus* also occur as commensals or pathogens of humans, whereas other causative species, such as *Str. uberis*, *Str. dysgalactiae* subsp. *Dysgalactiae* or *S. chromogenes*, are almost exclusively found in animals (Zadoks, et al., 2011). Environmental streptococci are predominant etiological agents of both subclinical and clinical forms of mastitis (Ferguson et al., 2007; Olde Riekerink et al., 2007). These pathogens are now emerging as one of the most frequent causes of bovine mastitis (Ebrahimi et al., 2008).

The subclinical form of mastitis is one of the most persistent and widely spread disease conditions of importance to milk hygiene and quality among dairy cattle (Mørk et al., 2007; Ogola et al., 2007; Jaime Romero et al., 2018).

Since the introduction of control measures to decrease the prevalence of contagious mastitis pathogens like *S. aureus* and *Str. agalactiae*, little progress has been achieved. The environmental pathogens *Str. uberis*, *Str. dysgalactiae*, and *Enterococcus* spp. (fecal streptococci) are ubiquitous in farms and cause major management difficulties. Although environmental streptococci can be cultured from milk virtually in all dairy herds, the epidemiology and dynamics of environmental streptococcal mastitis and the effects on milk yield and milk quality are poorly documented. According to Ramanauskienė et al. (2008), Fatma et al. (2019) and Gezehagn et al. (2020) the most frequently isolated causative agents of bovine mastitis are streptococci (41.59%) and staphylococci (20.35%)

On the other hand, mastitis is one of the major causes of antibiotic use in dairy cows. There is a variety of antimicrobials that are used for the prevention and treatment of mastitis. Therefore, resistance to antimicrobials is expected. Resistance of mastitis pathogens to antimicrobial agents is another well-documented challenge in dairy industry (Bradley 2007; Päivi Rajala-Schultz et al., 2021).

Several trials for development of bovine mastitis vaccine against the most common contagious and environmental pathogens have been reported (Amorena et al., 1994; Hwang et al., 2000; Dosogne et al., 2002). Also, numerous attempts of vaccination have been made employing live *S. aureus* strains, *Staphylococcus* peptidoglycan, toxoid, or preparation of killed *S. aureus*, Streptococcus species and avirulent *E. coli* strains. Most of these vaccines increased the rate of spontaneous recovery from the infection and lessened its severity but in many cases did not prevent the reoccurrence of new infections (Monaci et al., 2015).

Because the only available control measure of bovine mastitis is the use of expensive antibiotics and commercially available vaccines, the main objective in the present work was to prepare an inactivated polyvalent autogenous vaccine against the most prevalent bacterial pathogens recovered from clinical and subclinical mastitis cases in a dairy farm and to evaluate its immunizing efficacy and therapeutic potential.

MATERIAL AND METHODS

Samples

Milk samples: A total of 3608 quarter's milk samples were aseptically collected from 902 Friesian cows. The first few streams of milk were discarded and 15 to 20 ml of milk was collected separately from the four quarters of the udder into a sterile screw capped bottle. The collected milk samples were subjected to bacteriological examination.

Also, the somatic cell count and total immunoglobulins level were determined (Olde et al., 2007).

Blood samples: Blood samples were collected from cows in all groups before immunization and at 2 weeks intervals post immunization and/or antibiotic treatments. Serum was separated and kept frozen till examined.

Preparation of milk whey

Five mg of rennin were dissolved in 270 ml of sterile saline and one ml of this solution was added to 10 ml of defatted milk sample. After 30 min incubation at 37°C, the milk was centrifuged at 1000 xg for 20 min and then the supernatant (milk whey) was separated and kept frozen till examined (Frost and Tina, 1988).

Bacteriological examination

A loopful from the sediment of examined milk samples (pre-incubated at 37°C overnight and centrifuged at 3000 xg for 20 minutes) was streaked onto nutrient agar, blood agar and MacConkey agar plates. All plates were incubated at 37°C for 24-72 hrs and examined daily for bacterial growth. Single colonies were picked up and each was plated onto Edwards medium, Mannitol salt agar, EMB and Pseudomonas salt agar and incubated aerobically at 37°C for 24-72 hours for further identification. The isolated bacterial species were fully identified according to Quinn et al. (1994) and David et al. (2001).

Serotyping of the recovered bacterial isolates:

The recovered *E. coli* isolates were serotyped using commercial *Escherichia coli* antisera (Welcome Diagnostic Antisera). The isolated streptococcal species were serotyped using diagnostic reagent range from Oxoid (10 t/ch – P. 9939). These Kits were used for identifying Lancefield groups A, B, C, D and G of isolated streptococci. Dry spot kit (Staphytech plus - Oxoid. DR 100 M), and Latex slide agglutination test was used for identification of staphylococci.

Preparation of an inactivated polyvalent bovine mastitis bacterial vaccine: Bacterial species used: The following bacterial species, which were the most prevalent cause of mastitis in the examined dairy farms, were selected for preparation of an inactivated polyvalent autogenous mastitis vaccine. These include *S. aureus, Str. agalactiae*, *Str. dysgalactiae* and *E. coli* (serotype O111: K58 (B5).

Preparation of the bacterial antigen mass from obtained isolates: Briefly, the used bacterial species were separately inoculated into brain heart infusion broth and incubated at 37°C for 18 hours. The bacterial cells were harvested by centrifugation at 1800 xg for 20 min at 4°C and the supernatant was aseptically discarded. The cell sediment from these bacterial species was washed 3 times with sterile PBS and finally re-suspended in sterile PBS.

In the prepared polyvalent vaccine, the concentration of the bacterial components per vaccine dose was adjusted to 10^{10} CFU/5 ml of *S. aureus,* 4 x10^{9} CFU/5 ml of *Str. agalactiae,* 4 x10^{9} CFU/5 ml of *Str. dysgalactiae* and 1 x 10^{9} CFU/5 ml of *E. coli* serotype O111 using phosphate buffer saline (Hogan et al., 1995; Jose et al., 1997).

Inactivation of the bacterial cells using gamma irradiation: The bacterial mass of each of the 4 bacterial species was subjected to different doses of gamma irradiation trials to determine the minimum lethal dose according to Alur et al. (1998) and Ito (1998). The Minimum lethal dose of ionizing irradiation for the studied pathogens was found to be 4 kGy/min. The inactivated bacterial antigenic mass of the 4 included species in the abovementioned concentrations were mixed and freeze dried. The inactivated bacterial cells were mixed with Montanide, ISA-206 (SEPPIC, France) in a ratio 30:70 with continuous mixing Vincent et al. (2017). The mixture was gently dispersed using homogenizer till a stable oil emulsion with low viscosity was obtained.

Testing for sterility of the prepared vaccine: The vaccine preparations were inoculated on different bacteriological media, incubated for 3 days at 37°C and examined for the presence of any microbial growth (Vincent et al., 2017).

Vaccine efficacy trial

The prepared vaccine was evaluated in 6 groups of lactating cows (4 cows /group):

Experiment No. (1): It includes the first two groups which were clinically apparently normal cows and were used in evaluation of the immunizing efficacy of the prepared vaccine. (Vincent et al., 2017). The first group was kept unvaccinated and served as a control allover the study. The second group was immunized with the prepared vaccine.

Experiment No. (2): The other four groups of cows were included in this experiment. The cows in these 4 groups were suffering from subclinical mastitis and were used for evaluation of the therapeutic potential of the prepared vaccine 2ml /dose (Piepers et al., 2017). The first group (G1) was left untreated (neither antibiotics nor the vaccine). The second group (G2) was treated only with antibiotics (neomycin and penicillin). The third group (G3) was immunized with the prepared vaccine alone. The last group (G4) was treated simultaneously with both antibiotics and the prepared vaccine.

The vaccinated cows received two vaccinal doses at 2 weeks

Table 1: California mastitis test results for determination of the frequency of clinical and subclinical mastitis in dairy cows and percentage of quarters involvement.

Total numbers of cows examined	Clinical condition	Number of affected animals	%*	Total No. of quarters examined	Number of quarters affected	%
902	Animals with clinical mastitis	68	7.54	272	83	30.5
	Animals with subclinical mastitis	120	13.3	480	128	26.67

* Percentage was calculated according to the total number of examined cows.

Table 2: Incidence of pathogenic bacterial species in milk samples of cows with clinical or subclinical mastitis.

Isolated bacterial species	Clinical mastitis		Subclinical mastitis		Total number of isolates	%*
	No.	%	No.	%		
E. coli	20	29.41	35	29.2	55	29.26
S. aureus	13	19.12	30	25	43	22.87
S. dysgalactiae	10	14.7	24	20	34	18.09
S. agalactiae	11	16.2	20	16.6	31	16.49
S. uberis	5	7.3	3	2.5	8	4.26
K. pneumoniae	3	4.4	4	3.3	7	3.72
P. aeruginosa	1	1.5	4	3.3	5	2.65
E. fecalis	3	4.4	0	0	3	1.60
S. pyogenes	2	2.9	0	0	2	1.06
Total	68		120		188	100

*Calculated in relation to the total number of isolates.

Table 3: Types, number and percentage of *E. coli* serotypes recovered from milk samples of cows suffering from clinical and subclinical mastitis.

E. coli serotypes	Animals with clinical mastitis		Animals with subclinical mastitis		Total	
	No.	%	No.	%	No.	%
O111:K58 (B4)	9	45	12	34.3	21	38.2
O127:K63 (B8)	7	35	8	22.8	15	27.3
O 55:K59 (B5)	3	15	7	20.0	10	18.2
O125:K70 (B15)	1	5	5	14.3	6	10.9

Table 4: Total immunoglobulins (gm/dl) in serum and milk whey of apparently healthy cows immunized with the inactivated polyvalent mastitis vaccine.

Dairy cattle groups	Immunoglobulin's concentration (gm/dl) post immunization			
	In serum		In milk whey	
	2 weeks	4 weeks	2 weeks	4 weeks
Control (unvaccinated Cows)	1.48 ± 0.012	1.56 ± 0.034	2.83 ± 0.034	2.88 ± 0.043
Vaccinated cows	1.57 ± 0.036*	1.80 ± 0.049*	3.15 ± 0.018*	3.31 ± 0.030*

* Significant at P < 0.01 using t-test as compared with control.

interval and the vaccine was injected subcutaneously in the brachiocephalic muscle.

Blood and milk samples from cows in all groups were collected before and at 2 week interval post immunization and/or antibiotic treatment. Cows in all groups were monitored clinically allover the period of the study.

Statistical analysis

The obtained data were statistically analyzed using t-student test and One-way ANOVA according to Petrie and Watson (1999).

RESULTS

A total of 3608 quarter's milk samples were aseptically

Table 5: ELISA readings (OD) and corresponding titer of specific antibacterial antibodies in serum and milk whey of apparently healthy dairy cows immunized with the inactivated polyvalent bacterial vaccine.

		Reading	Control Non immunized Group (C.O.V.)*	Immunized apparently healthy cows (G I)			
				E. coli	*S. aureus*	*Str. agalactiae*	*Str. dysgalactiae*
Serum samples	2 weeks	OD	0.465	0.584	0.615	0.579	0.499
		Titer	0	1/160	1/160	1/160	1/80
	4 weeks	OD	0.462	0.642	0.662	0.656	0.534
		Titer	0	1/320	1/640	1/320	1/160
Milk whey samples	2 weeks	OD	0.415	0.640	0.618	0.557	0.508
		Titer	0	1/160	1/160	1/160	1/160
	4 weeks	OD	0.411	0.752	0.641	0.595	0.539
		Titer	0	1/640	1/320	1/320	1/320

Results were expressed as OD and representative antibody titer.
* C.O.V. = mean of reading (OD) of control groups + 2 x S.E.

collected form 902 Friesian cows, of which 68 animals (7.54%) suffered clinical mastitis and 120 cows (13.3%) proved to suffer subclinical form of mastitis using California mastitis test (Table 1).

Bacteriological examination of collected milk samples from cows with clinical and subclinical forms of mastitis:

Several bacterial species were recovered from the examined milk samples (Table 2). The most prevalent bacterial species were *E. coli* (29.26%), *S. aureus* (22.87%), *Str. dysgalactiae* (18.09%) and *Str. agalactiae* (16.49%). Several other bacterial species were recorded at a lower rate including *Str. uberis, K. pneumoniae, P. aeruginosa, E. fecalis* and *Str. pyogenes*. Among the isolated *E. coli* isolates, the serotype O111:K58 (B4) was the most prevalent (38.2 %) (Table 3).

Experiment No. (1)
The immunizing efficacy of the prepared polyvalent inactivated mastitis vaccine in apparently healthy lactating cows:
Clinical signs and post vaccination reaction: Cows vaccinated with the prepared polyvalent inactivated mastitis vaccine manifested a mild vaccination reaction at the injection site in a form of swelling that subsides within 4 - 6 days post vaccination. Also, mild rise in rectal temperature (from 0.5 to 1°C) of the injected animals was recorded one hour after the time of injection and remained for 2 days (data not shown). There was no significant reduction of milk yield following immunization with the prepared vaccine. All cows in these groups did not develop clinical mastitis during the whole observation period that extends up to 20 weeks post vaccination.

Immunoglobulin concentration: As shown in Table 4, significant ($P < 0.01$) increase in immunoglobulins concentration (g/dl) was recorded at the 4th week post immunization of the apparently healthy cows, which reached 1.80 ± 0.049 and 3.31 ± 0.030 g/dl in serum and milk whey, respectively, as compared to 1.56 ± 0.034 and to 2.88 ± 0.043 in serum and milk whey from the unvaccinated control cows.

ELISA RESULTS

As shown in Table (5), the highest level of antigen-specific antibodies against the immunogenic components of the prepared vaccine was recorded in serum and milk whey at the 4th week post immunization in cows treated with the vaccine alone. It reached 1/320 against *E. coli* (O_{111}) and *Str. agalactiae*, 1/640 against S. *aureus* and 1/160 for *Str. dysgalactiae*, in serum while it reached 1/640 against *E. coli* and 1/320 against *S. aureus*, *Str. agalactiae* and *Str. dysgalactiae* in milk whey.

Experiment No. (2):
The therapeutic potential of the inactivated polyvalent bacterial mastitis autogenous vaccines in cows suffering from subclinical mastitis:

1. Effect of vaccination on the somatic cell count (SCC): The analysis of SCC in milk samples collected before and 4 weeks post vaccination in cows suffering from subclinical mastitis is shown in Fig. (1). Significant ($P < 0.01$) reduction in the SCC after 4 weeks post vaccination and/or antibiotic treatment was recorded. In the antibiotic treated group the SCC decreased from 35 x 10^4 to 27 x 10^4. In the vaccinated group the SCC decreased from 34 x 10^4 to 24 x 10^4. In the third group that was treated with both the antibiotic and the vaccine, the SCC decreased significantly form 30 x 10^4 to 18 x 10^4.

2. Effect of vaccination on the immunoglobulin concentration: As shown in Fig. (2) and (3), significant ($P < 0.01$) increase of total immunoglobulins in the serum and milk whey was recorded in cows immunized with the prepared vaccine alone. In non-immunized cows and in cows treated with the antibiotics or with the vaccine and antibiotics no significant ($P > 0.05$) increase of total globulins in serum

and milk whey was recorded.

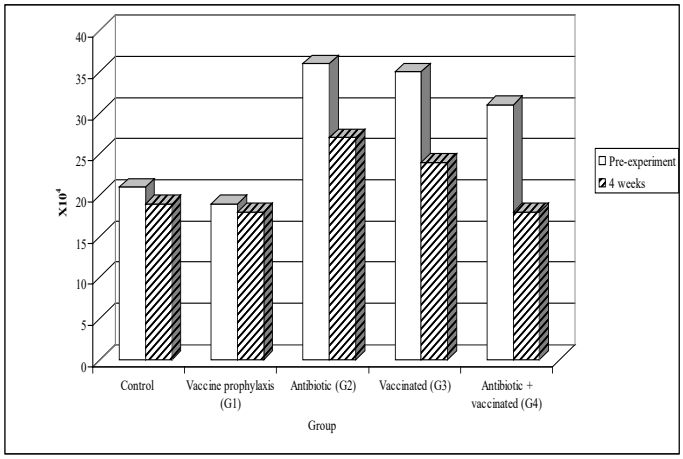

Figure 1: Somatic cell count (SCC)/ml of milk samples in differently treated groups of dairy cattle pre-experiment and at 4 weeks post immunization.

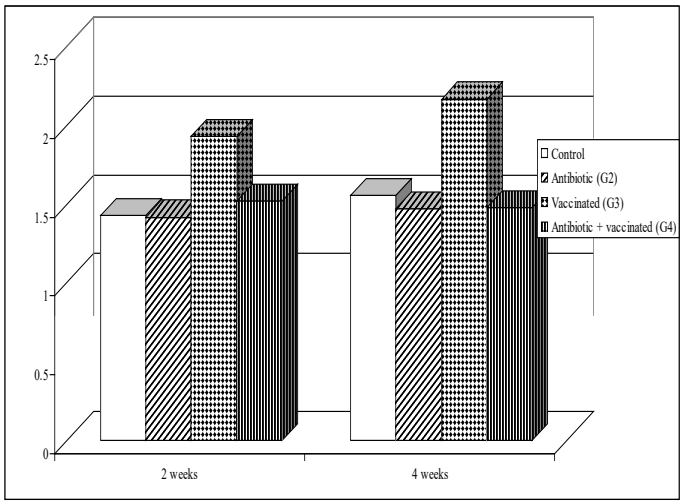

Figure 2: Total immunoglobulins (gm/dl) in serum of differently treated groups of cows with subclinical mastitis.

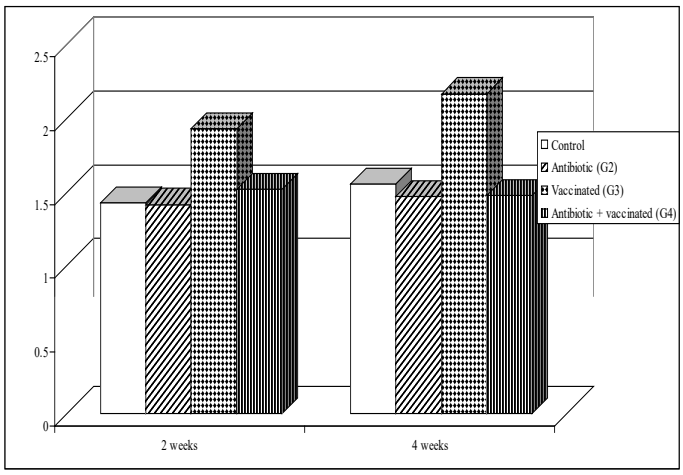

Figure 3: Total immunoglobulins (gm/dl) in milk whey of differently treated groups of cows with subclinical mastitis.

DISCUSSION

Despite the implementation of standard mastitis control programs, mastitis is still economically among the most important diseases of dairy animals. Approaches to enhance the cow's immunity to prevent mastitis and thus minimize use of antibiotics have gained considerable attention. Yet, for a variety of reasons, vaccines developed for the prevention and control of mastitis have achieved only limited success. The multiplicity of pathogens capable of causing mastitis and insufficient knowledge of mammary gland immunology, bacterial virulence factors and mechanisms of pathogenesis are factors that have hindered development of effective mastitis vaccines. Several attempts have been made to develop and evaluate monovalent and polyvalent vaccines against the most prevalent mastitis pathogens like *S. aureus, Str. agalactiae, E. coli* and *Str. uberis*. In general, the commercially available *S. aureus* vaccines although have a limited to moderate ability to prevent new infection, they could enhance the spontaneous cure rates and reducing chronic infections (Piepers et al., 2012; Schukken, et al., 2014).

The first part of the present work was designed to determine the most prevalent bacterial pathogens that underline cases of clinical or subclinical mastitis in cattle. *E. coli*, *S. aureus*, *Str. agalactiae* and *Str. dysgalactiae* were the most common etiological agents recorded. Therefore, a custom designed inactivated polyvalent autogenous vaccine was prepared from these four species and its immunizing and immunotherapeutic potential was determined. The selected bacterial species were grown, mixed and inactivated using gamma radiation. The minimum inactivating irradiation dose was determined and proved to be 4 krad/min (kGy). Similarly, Hammad et al. (1998) reported that an irradiation dose of 4 kGy greatly reduced the microbial count and extend the shelf lifetime of foods. Variation among the intensity of irradiation doses (2-10 kGy) required for microbial inactivation has been reported by several authors (Cho et al., 1986; Leslie, et al., 2008; Fahmida et al., 2013, Wilmar et al., 2019; Eve et al., 2020). The variation of the intensity of irradiation required for inactivation was found to be influenced by several factors, among other, the microbial species involved and the nature of the irradiated material (solution *vs.* dried form) (Andreia et al., 2016; Yifan et al., 2018).

The immunizing and immunotherapeutic potential of the inactivated polyvalent bacterial mastitis autogenous vaccine were determined in a group of cattle in a dairy farm from which the above-mentioned bacterial species were recovered. Determination of the immunizing potential of the prepared vaccine was done in apparently healthy cows, while the immunotherapeutic efficacy was evaluated in cows suffering subclinical mastitis. Slight swelling at the

site of the injection was observed in all vaccinated cows. It continued for 2-6 days post-injection and returned to normal after that. This swelling can be considered as a normal reaction to the injected vaccine. The vaccine was prepared using sterile distilled water free from pyrogenic and toxigenic elements. Compared with the control non-immunized cows, an increase of the rectal temperature (0.5 to 1 °C) was noticed in the injected cows. It persisted for 2 days and became normal after that. The skin reaction and the elevation of temperature, however, can be attributed to the previous sensitization of the injected animals to one or more of the antigenic components of the used vaccine (Dosogne et al., 2002). No significant decrease in milk yield in the days immediately following the immunization was recorded.

The immunizing efficacy of the prepared vaccine was determined using two parameters; first the titer of the specific antibodies developed against microbial components of the vaccine and secondly the level of total immunoglobulins in sera and milk whey of the immunized cows. In the present work, evaluation of the vaccine was limited to its immunizing efficacy only, where the protective efficacy of the vaccine could not be determined. It was impossible for us to run intramammary experimental infection of the immunized cows.

Investigating the effect of immunization of cows with the prepared vaccine on the serum level of total immunoglobulins at 2- and 4-weeks post immunization revealed an increase in the immunoglobulins level in the immunized cows, which reached 1.57 and 1.80 g/dl as compared with 1.48 and 1.56 g/dl in control non-immunized group. Similarly, the level of total immunoglobulins in milk whey of the immunized cows reached to 3.15 and 3.31 g/dl at 2- and 4-weeks post immunization compared to 2.83 and 2.88 g/dl in the control non-immunized cows. Similar result was recorded by Gilman et al. (1991) and Tomita et al. (1998).

Immunization of cows with the prepared inactivated polyvalent bacterial mastitis vaccine variably stimulated the production of specific antibodies against the bacterial components of the vaccine both in the serum and milk whey. Two weeks post immunization a titer of 1/160 of antibodies specific to *E. coli*, *S. aureus*, *Str. agalactiae* and a titer of 1/80 against *Str. dysgalactiae* was measured in sera of the immunized cows. Four weeks post immunization (2 weeks after the booster dose) a significant increase was reported against *S. aureus*, where the antibody titer reached 1/640. Also increase in the anti- *E. coli* and anti- *Str. agalactiae* antibody titer reaching 1/320 was measured. An increase of 1/160 was recorded against *Str. dysgalactiae*. Similar results were reported by several authors using monovalent or polyvalent vaccine (Amorena et al., 1994; Zuhair, 2017). Also, Tomita et al. (1995 and 1998) and Hogan (1999) reported significant increase in IgG antibody titer against *E. coli* in serum of cattle vaccinated with *E. coli* j5 vaccine.

In the milk whey of the immunized cows, the anti-bacterial antibodies detected after two weeks from the immunization reached 1/160- and double-fold increase was measured two weeks following the booster dose. Similar results were reported by Tomita et al. (2000). However, it was observed that the level of anti *E. coli* antibodies was higher in milk whey than the level of anti *S. aureus* antibodies in contrast to what was recorded in the serum of the immunized cattle. This might be attributed to the immunoglobulin classes or subclasses produced against the different bacterial component of the vaccine and perhaps those produced against *E. coli* were of IgG1 isotype that are more secretable in milk compared to other immunoglobulin subclasses that are found in high concentration in blood (Amorena et al.,1994). Tomita et al. (1998) reported also that vaccination of heifers with *S. aureus*, *Str. agalactiae* and *Str. uberis* on days 55 and 45 prior to calving induced significant increase in the blood serum level of IgG1, slight increase in IgG2 content (as a percent of total immunoglobulins) and decrease in IgM content for 1 month after calving.

In spite of the fact that specific antibodies against the bacterial components of the vaccine was detected in blood and milk of immunized cows and none of the vaccinated cows developed clinical mastitis during the whole observation period (20 weeks), no clear statement could be concluded on the protective nature of the prepared vaccine. Evaluation of the protective efficacy required the intramammary experimental infection of cows with the pathogenic bacteria in an experimental model,

In the second experiment the immunotherapeutic potential of the prepared vaccine was evaluated in cows with diagnosed subclinical form of mastitis from which the vaccine components of bacterial species were isolated. All cows in the 3 treated groups did not proceed to clinical mastitis during the whole observation period that extended for 20 weeks. It was observed also that animals treated with the vaccine alone developed significant increase of specific antibodies against the microbial components of the used vaccine. However, in the group treated with the vaccine and antibiotics lower level of antibody response was measured. This might be attributed to possible immunosuppressive effect of the used antibiotics.

Analysis of the result of SCC determined 2 and 4 weeks post treatment revealed significant reduction after 4 weeks from start of the treatment in the three differently treated groups. However, in group that was treated with the anti-

biotic and the vaccine, the reduction of the SCC count was highly significant and the cell number returned to figures similar to those counted in the apparently normal controls. These results are in agreement with those reported by Jose et al. (1997).

Although the success of mastitis vaccine is difficult to define, but its use for therapeutic purpose proved effective as it stops the progress of subclinical cases to proceed to clinical mastitis and also was associated with reduction of the SCC to its normal level. Similar result was recorded by Hogan et al. (1995) who reported rapid recovery and reduction of clinical signs of experimental *E. coli* mastitis through vaccination.

Spontaneous recovery and significant reduction of the microbial CFU /ml of the examined milk samples was recorded in the present work. Leitner et al. (2004) reported that 30% of the *S. aureus* infected cows when vaccinated with a commercial *S. aureus* vaccine remained infection free till the end of 348-day trial, which suggest a curative role of this vaccine.

A recent study made by Athar (2007) demonstrated that locally prepared polyvalent bacterin mastitis vaccines (containing killed *S. aureus, Str. agalactiae,* and *E. coli* with various adjuvants) not only prevented new infections but also cured existing infections of these organisms in dairy buffaloes. Similar observations were made with locally prepared *S. aureus* vaccines (live attenuated vaccine, plain bacterin, dextran sulphate adjuvanted bacterin and oil-adjuvanted bacterin (Ahmed, 2010). Also Hwang et al. (2000) reported the therapeutic efficacy of autogenous *S. aureus* vaccines in Korea. They investigated therapeutic efficacy of an autogenous *S. aureus* toxoid-bacterin in lactating cows suffering from subclinical *S. aureus* mastitis and proved that the autogenous toxoid-bacterin treatment against *S. aureus* subclinical mastitis in lactating cows increase the cure rate of existing infections, reduce their severity and also prevent occurrence of the new infections. It is interesting to note that most *S. aureus* vaccines developed for the prevention of infection act as better curative than preventative agents (Clegg et al., 2021).

CONCLUSION

The obtained results indicated that the prepared polyvalent inactivated autogenous vaccine and the immunization program including subcutaneous injection of two vaccine doses, each of 2ml/animal at 2 weeks interval in the brachiocephalic muscle is efficacious in the reduction of the incidence of intramammary infection due to *S. aureus, Coliforms, Str. agalactia* and *Str. dysgalactiae* in cows. Also, immunization significantly reduces the severity of the symptoms and causes a significant increase in the spontaneous cure rate of cows suffering subclinical mastitis where no case developed acute mastitis.

ACKNOWLEDGMENTS

This work is supported by the Science, Technology and Innovation Funding Authority (STDF) ID 27811.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

NOVELTY STATEMENT

Institutional Animal Care and Use Committee at Central Laboratory for Evaluation of Veterinary Biologics approved the research manuscript and it has been reviewed under our research authority and is fulfilling bioethical standards.

AUTHORS CONTRIBUTION

Experiments were designed by NGS and MSA, and the experiments were performed by HAK, NMA, and MSA. Data analysis was accomplished by NMA. ISY and MSA. The manuscript was written by HAK and MSA.

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