Efficacy of a locally prepared bovine mastitis vaccine

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A B S T R A C T

The objective of the present work is to prepare and evaluate the effectiveness of a locally prepared polyvalent vaccine against mastitis from the most common causes of mastitis. *Staphylococcus aureus, Streptococcus agalactiae* and *Escherichia coli* were the most prevalent bacteria recovered from clinical and subclinical mastitis. MontanideISA-206 adjuvanted inactivated polyvalent vaccine containing the three strains was prepared. Twenty pregnant cows were inoculated intramuscularly with the prepared polyvalent vaccine two months prior to calving and boostering at day 21 from the primary injection. Serum samples from vaccinated and non-vaccinated cows were collected at the 1st, 3rd, 8th, 12th, 16th, 20th and 24th weeks post vaccination and evaluated immunologically using ELISA. The results showed that immune response was significantly higher in the vaccinated group than that of controls. These results could be indicated the safety and effectiveness of the vaccine in reduction of incidence and severity of clinical cases of mastitis but further studies should be done to elucidate the possibility of field application and effectively.

Keywords: mastitis, vaccine, *S. aureus, S. agalactiae, E. coli*.

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1. INTRODUCTION

Mastitis is one of the most important diseases in dairy cows throughout the world, and is responsible for significant economic losses to the dairy industry due to loss in milk production, discarded abnormal milk, degrading milk quality and price due to high bacterial or somatic cell count, high treatment cost, increased labor costs, increased risk of subsequent mastitis, herd replacement and problems related to antibiotics residues in milk and its products (*Seegers et al. 2003*). Mastitis can be caused by a series of pathogens, differentiated into two broad categories: those causing contagious mastitis such as *Staphylococcus aureus* (*S. aureus*), *Streptococcus agalactiae* (*St. agalactiae*) which are widespread from the infected quarters, primarily during milking (man hands, milking machines), and those causing environmental mastitis such as *Streptococcus uberis, Streptococcus dysgalactiae, Escherichia coli*(*E.coli*) which are present in the environment (bedding, flooring, droppings) and generally transmitted in any time of cow's life: during milking, between milking and during the dry period, especially at first calving in heifers (*Radostits et al., 2000*). *Elbably et al., (2013)* stated that the most prevalent causes of mastitis are *S. aureus* (25.8%) followed by *E.coli* (18.7 %) and *Streptococcus agalactiae* (11.8 %) in addition, *Rafik et al., (2014)* found that the most prevalent pathogens were *E. coli* (25.5%), *S. aureus* (14.8%) and *St. agalactiae* (12.7%). Antibiotic treatment is the method most often used to fight mastitis, because of milk antibiotic residue affects food safety due to possible induction of drug resistance in bacteria; there is a regulatory pressure to justify the use of antimicrobials to control mastitis in dairy cattle (*Hu et al., 2010*). Vaccines would be a logical and promising approach to prevent mastitis in food production animals (*Talbot and Lacasse, 2005*).Traditional *S. aureus* mastitis vaccines

have included killed or attenuated bacteria, toxoids and cell wall extracts from selected laboratory or field strains (*Watson, 1992 and Watson et al., 1996*). Commercially available *S. aureus* mastitis bacterins are Lysigin (Boehringer Ingelheim Vetmedica, Inc) and Startvac® (Hipra, Inc., Spain). In Egypt the only available vaccine is Lysigin which aid in prevention of mastitis caused by *S. aureus*. Vaccination should be done with more than one organism which conceivably is more pragmatic **(***Hill, 1990; Calzolari et al., 1997 and Yancey, 1999*). Adjuvants when used in vaccines enhance immune response by augmenting antigenic properties of an antigen (*Jolles and Paraf, 1973*). Montanide ISA-206 is extremely inexpensive to use mineral oil adjuvant. The vaccine containing Montanide adjuvants are reported to have no toxic effect even after booster dose (*Barnett et al., 1996*).

So, the plan of this study was to prepare a polyvalent inactivated oily mastitis vaccine containing local strains of *S. aureus*, *St. agalactiae* and *E.coli* which were isolated from clinical and subclinical mastitic cases, and immunologically evaluation of levels of antibodies in serum of vaccinated cows.

2. 2. MATERIAL AND METHODS

2.1. Milk samples

Milk samples were collected from cows under aseptic conditions in a sterile screw caped bottle. The samples were labeled to identify each particular quarter. The milk samples were kept in ice container and transported as soon as possible to the laboratory.

2.2. Isolation, identification and bio-characterization of mastitis causative organisms

Bacteriological examination was applied on each milk sample and isolation of the causative organism was done according to Quinn et al., (1994). Biochemical identification of isolated organisms was made using different API systems (Biomerieux–France).

For Enterobacteriaceae API 20E reagent kit, for Staphylococcus species API-Staph Kit and for streptococcus species API 20 STREP. Pure growth was maintained on Tryptic soya broth containing 20% glycerol at -70°C for further use.

2.3. Vaccine preparation

Isolates of *S. aureus* and *St. agalactiae* were grown in brain-heart infusion broth at 37 °C for 24 hours. The cells were inactivated with formalin (0.4%/ vol.) according to *Giraudo et al. (1997).E. coli* strain was seeded into Tryptic soy broth medium and incubated at 37°C for 24 hours. Formalin (0.3% /v) was added to inactivate the bacteria according to *Acres et al. (1979).* Cultures of each strain was adjusted to contain 1x 10^{10} colony forming unit (cfu) per ml of *S. aureus*, 4 x 10^9 cfu/ml of *St. agalactiae* and 1 x 10^9 cfu/ml of *E. coli*. Equal volumes of each bacterial culture were mixed together and Montanide® ISA 206 (Seppic, France) was added in equal volume.

2.4. Animals and experimental design

A total of 400 dairy cows belonged to different farms in Cairo, Egypt were examined over one year. Mastitis was diagnosed using California Mastitis Test (CMT)according to *Schalm et al., (1971)* and results of clinical inspection of the udder**.** Forty five Holstein cows 2-4 years of age, with no clinical udder abnormalities, not previously received any vaccine against mastitis and free from antibodies against *S. aureus, St. agalactiae* and *E. coli* were used for evaluation of the prepared vaccine. These cows were divided into 3 groups (A, B and C).Twenty pregnant cows in group (A) were inoculated intramuscularly with 5 ml/cow of the prepared vaccine two months prior to calving then a booster dose of 5 ml/cow at day 21 of the primary injection. Five cows in group (B) were inoculated with double dose (10 ml/cow) intramuscularly and kept under daily observation for 14 days for any vaccine reaction (safety test). While twenty cows in group (C) were kept as non-vaccinated control group.Pooled serum samples were

collected at the 1st, 3rd, 8th, 12th, 16th, 20th and 24th weeks post vaccination.

2.5. Quality control testing of the prepared vaccine

2.5.1. Sterility test

Testing the freedom of the prepared vaccine from foreign contaminants (aerobic and anaerobic bacteria and fungi) was carried out according to*OIE (2013).*

2.5.2. Safety test

Safety of the prepared vaccine was tested according to *OIE (2013)* through inoculation of double dose intramuscular in each of five cows which kept under daily observation for 14 days.

2.5.3. Determination of immune response to the prepared experimental vaccine

ELISA was performed as previously described by *Leitner et al., (2000).*Each serum sample was tested for *S. aureus, St. agalactiae and E. coli*antibodies. In brief, 96-well immunoplates (Nunc-Immuno Plate Maxi SorbTM)) were coated with killed sonicated *S. aureus, St. agalactiae and E. coli* as antigenand incubated 2 hours at 37 ◦C and overnight at 4 ◦C. The coating solution was then discarded and the plates were blocked with bovine serum albumin 3% at 37 ◦C for one hour. After washing plates three times with PBS-Tween (PBS 0.1M pH 7.4, 0.5% Tween 20), tested serum (1:200) was added in duplicate, incubated for 1 h at 37 ◦C then affinity-purified goat anti-Bovine IgG (g) chain peroxidase conjugate (1:2000) (KPL company) was added. The bound antibodies were detected by adding TMB peroxidase substrate (Kirkegaard and Perry Laboratories). Plates were read in a microplate auto reader at 450nm. Negative serum samples from uninfected cows were used to calculate the cut-off value of the indirect ELISA. Cut-off = Mean of Negatives + (3 x standard deviations of Negatives). Samples was considered as positive if the OD values were higher than the cut-off value, otherwise, the sample was regarded as negative if the OD was below the cut-off value *(Tong et al., 2014).*

2.6. Statistical analyses

Results of ELISA test in table (1) were analyzed and compared with parametrical correlation using Student's t test *(Sendecor, 1971).*

3. RESULTS

Four hundred dairy cows were examined for the presence of clinical and subclinical mastitis. The Incidence of clinical and subclinical mastitis in examined dairy cows and bacterial isolation from positive cases are shown in table (1), where incidence of clinical and subclinical mastitis at cow's level were 7% (28/400) and 36% (144/400) respectively. On the other hand, bacterial pathogens were found in 92.8% of the clinical mastitis (26/28) and in 88.9% of the subclinical mastitis samples (128/144).

On regarding to the incidence of clinical and subclinical mastitis in twenty control non-vaccinated dairy cows is shown in table (2), it was found that one cow out of the control cows (20) suffered from clinical mastitis and three cows suffered from subclinical mastitis. *S. aureus* was isolated from the clinical mastitis control cows while *S. aureus*, *Str. agalactiae* and *E.coli* were isolated from the subclinical mastitis control cows. On the other side, in the vaccinated group only one cow showed subclinical *S. aureus* mastitis as shown in table (3).

The results of the sterility test showed that the prepared vaccine was proved to be free from any extraneous contaminants (aerobic and anaerobic bacteria and fungi).

No general adverse reactions to the vaccine were observed in vaccinated or re-vaccinated cows throughout the entire experimental period indicating the safety of the prepared vaccine.

Results of ELISA (expressed as mean optical density) against *S. aureus, Str.*

agalactiae and E. coli in serum of vaccinated and control non-vaccinated cows are shown in Table (4) and Fig. (1, 2 and 3). The results of the immune response against the three antigens showed the same pattern where the mean OD gradually increased till reached the peak (3.203, 2.850 and 3.482 respectively) at 8th week post first dose of vaccination then decreased gradually till reached the lowest values (1.311, 1.324 and 1.092 respectively) at 24th week post first vaccination. The results of the vaccinated group were compared with those of control by using T-test, it was noticed that the mean OD of the vaccinated groups increased significantly than those of control groups.

Table (1): Incidence of clinical and subclinical mastitis in examined dairy cows

No. of examined cows	Clinical mastitis*				Subclinical mastitis**			
	Milk samples from clinically infected cows		Bacterial pathogens isolated		Milk samples from apparently healthy cows		Bacterial pathogens isolated	
	No.	%	No.	%	No.	%	No.	%
400	28	7	26	92.8	144	36	128	88.9

* Hot, hard sensitive udder that is acute painful to the animal with changes in composition. ** No visible changes in appearance of udder and/or the milk but the milk was positive for California Mastitis Test (CMT).

Table (2): Incidence of clinical and subclinical mastitis in control non-vaccinated dairy cows

No. of examined cows	Clinical mastitis*				Subclinical mastitis**			
	Milk samples from clinically infected cows		Bacterial pathogens isolated		Milk samples from apparently healthy cows		Bacterial pathogens isolated	
	No.	%	No.	%	No.	%	No.	%
20	1	5	*1 (S. aureus)*	100	3	15	*3 (S. aureus St. agalactiae E. coli)*	100

Table (3): Incidence of clinical and subclinical mastitis in vaccinated dairy cows

Number of examined cows	Clinical mastitis*				Subclinical mastitis**			
	Milk samples from clinically infected cows		Bacterial pathogens isolated		Milk samples from apparently healthy cows		Bacterial pathogens isolated	
	No.	%	No.	%	No.	%	No.	%
20	0	0	0	0	1	5	S. aureus	100

Table (4): The mean serum Optical Density (OD) values in dairy cows vaccinated with polyvalent inactivated Staphylococcus aureus, Streptococcus agalactiae and Escherichia coli vaccine measured by ELISA

WPV / Antigens	First dose	booster dose 3WPV	8WPV	12WPV	16WPV	20WPV	24WPV
Staphylococcus aureus antigen	0.042	3.024*	3.203*	2.866*	2.337*	1.426*	1.311*
Non vaccinated	0.433	0.421	0.445	0.411	0.453	0.427	0.439
Streptococcus agalactiae antigen	0.039	2.221*	2.850*	2.773*	2.189*	1.370*	1.324*
Non vaccinated	0.201	0.211	0.209	0.218	0.206	0.217	0.221
Escherichia coli antigen	0.037	3.264*	3.482*	2.778*	2.312*	1.143*	1.092*
Non vaccinated	0.331	0.347	0.351	0.341	0.323	0.353	0.351

WPV weeks post first dose
* Significant at P > 0.05
Cut-off values (S. aureus = 0.48, St. agalactiae = 0.23, E. coli = 0.38)

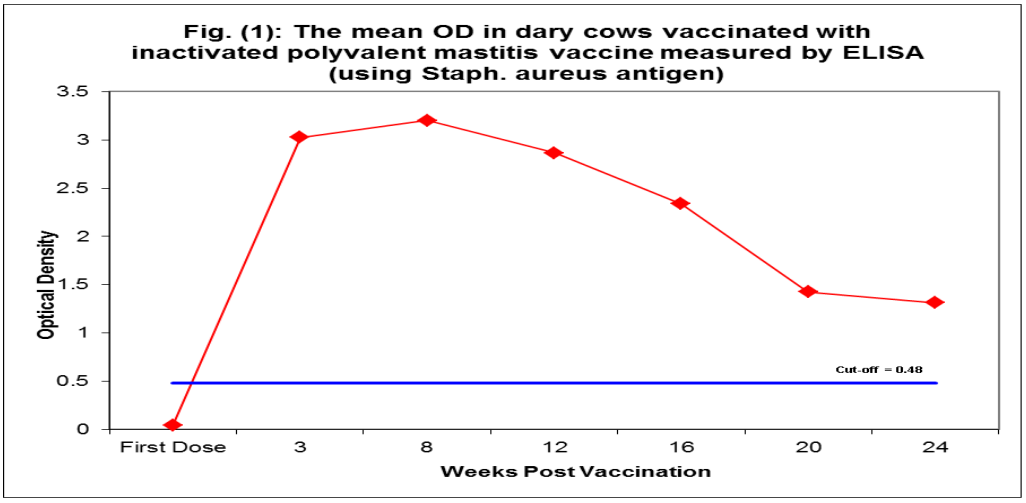

Fig. (1): The mean OD in dary cows vaccinated with inactivated polyvalent mastitis vaccine measured by ELISA (using Staph. aureus antigen)

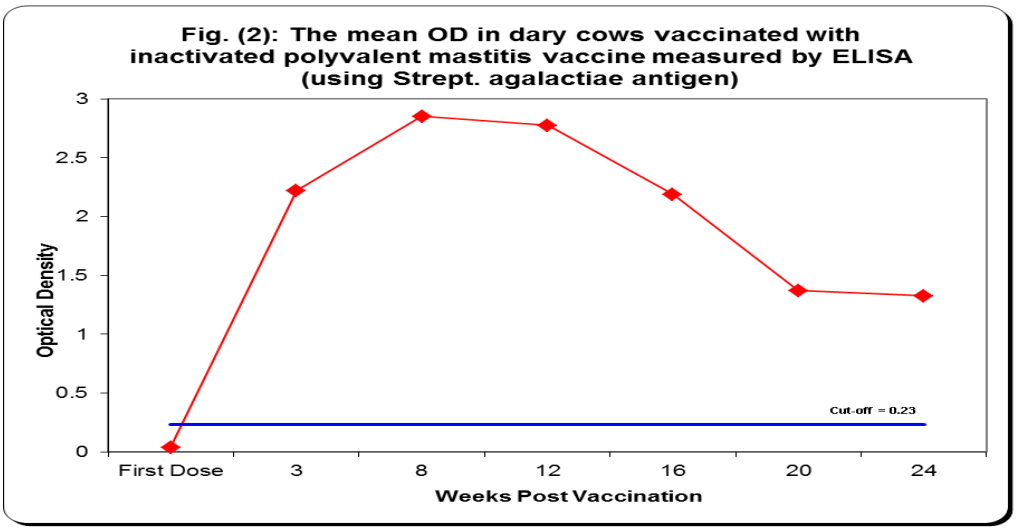

Fig. (2): The mean OD in dary cows vaccinated with inactivated polyvalent mastitis vaccine measured by ELISA (using Strept. agalactiae antigen)

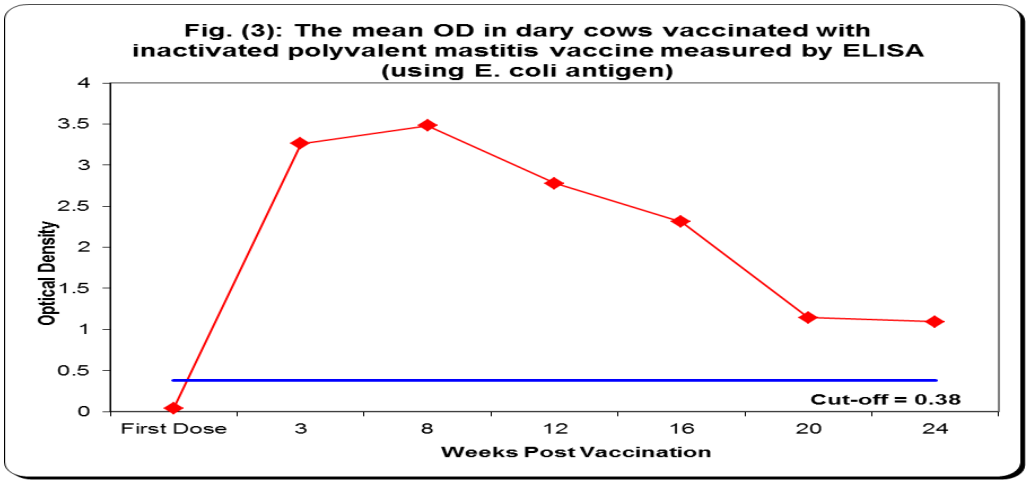

Fig. (3): The mean OD in dary cows vaccinated with inactivated polyvalent mastitis vaccine measured by ELISA (using E. coli antigen)

4. 4. DISCUSSION

A total of 400 dairy cows were examined for clinical and subclinical mastitis. The results showed that cows with clinical mastitis were exhibited hotness, redness, edema, enlargement and hardness of the affected quarter. This observation is in accordance with that represented by *Nickerson (1985)* who found similar clinical findings of mastitis. CMT is a screening test for subclinical mastitis that can be used easily (*Leslie et al., 2002*). It is widespread used in dairy fields and recommended (*Shitandi and Kihumbu, 2004*) and considered as rapid and characteristics indicator for the infection of mammary gland (*Al-Anbari et al., 2006*). Incidence of clinical and subclinical mastitis at cow's level were 7% (28/400) and 36% (144/400) respectively as shown in table (1). The obtained results come nearly in agreement with result of *Ahmed (2006)* who stated that the percentage of clinical mastitis was 9.66% and *Lakew et al. (2009)* who recorded 38.1% of dairy cows have subclinical mastitis. Meanwhile, higher incidence was recorded by *Rafik et al. (2014)* who determined the percentage of subclinical mastitis as 56.3% while that of clinical mastitis as 13.3%.

Bacterial pathogens were found in 92.8% of the clinical mastitis (26/28) and in 88.9% of the subclinical mastitis samples (128/144) as shown in table (1). The clinical and subclinical mastitis without isolation of the causative agent may be attributed to failure of organism isolation by the employed cultural techniques (selective media for mycoplasmas, haemophilus and fungi were not employed). This reason was in agreement with that proposed by *Ismail andHatem (1998).* Also, (10-20%) of cows sampled for bacterial culture based on CMT score will have no growth due to a number of factors including short lived infections that have been cleared by the cow or infections that are characterized by intermittent shedding of bacteria (*S. aglactiae, S. aureus* and *Mycoplasma spp.*).

Bacterial isolation and biochemical identification revealed three dominating bacterial species which were *S. aureus* (45 %), *S. agalactiae* (26%)and *E. coli* (12%). In this respect *Gonzalo et al. (2002)* stated that *S. aureus, S. agalactiae* and *E. coli* are the most common etiological agents involved in subclinical and clinical cases of mastitis in dairy cows. Also, *Abdel-Rady and Sayed(2009)* found that the most frequently major causative isolated agents were *S. aureus, St. agalactiae* and *E. coli* from the positive California mastitis test (CMT) samples with prevalence 52.5%, 31.25% and 16.25%, respectively and *Elbably et al. (2013)* stated that the most prevalent causes of mastitis are *S. aureus* (25.8%) followed by *E.coli* (18.7 %) and *St. agalactiae* (11.8 %). Moreover, *Rafik et al. (2014)* found that the most prevalent pathogens were *E. coli* (25.5%), *S. aureus* (14.8%) and *St. agalactiae* (12.7%).

Talbot and Lacasse (2005) claimed thatvaccines would be a logical and promising approach to prevent mastitis in food production animals. Moreover, it is well established that immunosuppression of cows during the pre-parturient period considerably increases the incidence of mastitis in early lactation and many attempts have been made to improve the resistance of animals to intramammary infections during this period *(McDougall et al. 2009 and Middleton et al. 2009).*

The results of the sterility test showed that the prepared vaccine was proved to be free from any extraneous contaminants (aerobic and anaerobic bacteria and fungi).

No general adverse reactions to the prepared vaccine were observed in vaccinated or re-vaccinated cows throughout the entire experimental period indicating the safety of the prepared vaccine. Montanide adjuvanted vaccine has been shown to be less irritant to tissue as compared to the traditional Freund’s

adjuvanted vaccines (*Cook et al., 1990*). Also, the vaccine containing Montanide adjuvants are reported to have no toxic effect even after booster dose (*Barnett et al., 1996*).

One of the basic criteria for evaluation of vaccine efficacy is to assess its ability to reduce the prevalence and incidence of the disease. An effective mastitis vaccine should reduce the prevalence and incidence of mastitis caused by that particular organism against which vaccine is administered. In the present study, Table (2) showed that one cow out of the control cows (20) suffered from clinical mastitis and three cows suffered from subclinical mastitis. S. *aureus*, *Str. agalactiae* and *E.coli* were isolated from the clinical and subclinical mastitic control cows. On the other side, in the vaccinated group (table 3) only one cow showed subclinical *S. aureus* mastitis. These differences could be considered as an indicator for potential protective effect of the prepared vaccine. Reduced severity of symptoms is probably mediated via antibodies neutralizing the *S. aureus* toxins, and this effect may be the easiest to generate when *S. aureus* immunization is used (*Rainard and Poutrel, 1991* and *Watson, 1992*). The reduction in number of clinical mastitis cases has also been reported by *Tollersrud (2002).* Also, *Norcross and Kenny (1994)*reported fewer new infections in vaccinated cows as compared to control and *Nordhaug et al.(1994); Giraudo et al. (1997)* and *Nickerson et al. (1997)*reported reduction in point prevalence and incidence rate of mastitis in vaccinated animals.

Mean Optical Density (OD) against S. *aureus*, *Str. agalactiae* and *E.coli* in serum of vaccinated and non-vaccinated cows is shown in Table (4). The results of the three antigens showed the same pattern where the mean OD gradually increased till reached the peak at 8th week post first dose of vaccination then decreased gradually till reached the lowest values at 24th week post first vaccination. The results of the vaccinated group were compared with those of control by using T-test, it was noticed that the mean OD of the vaccinated groups increased significantly than those of control groups. Same results were obtained by *Hogan et al. (2005)*, *Pellegrino et al. (2008)* and *Perez et al. (2009).* As shown in Table (4) and Fig. (1, 2 and 3), it was noticed that all serum samples during the period 3 WPV till 24 WPV were positive, where the OD were above their cut-off values (*S. aureus* = 0.48, *St. agalactiae* = 0.23, *E. coli* = 0.38) (*Tong et al., 2014*).

These results could be indicated the safety and effectiveness of the vaccine in reduction of incidence and severity of clinical cases of mastitis but further studies should be done to elucidate the possibility of field application and effectively.

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