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Field Trial to Evaluate Vaccine and Antibiotic for Control of *Staph. aureus* Mastitis in Dairy cattle, Egypt

Ramy F. Ghobrial[1], Mohamed A. EL Beskawy[2], Mohamed M. EL Diasty[1], Verginia M. Farag[3], Mohamed I. Eissa[4]

[1]Animal health research institute, Mansoura provincial laboratory, Egypt.
[2] Department of Internal Medicine, Infectious and fish diseases, Faculty of Veterinary Medicine, Mansoura University, Mansoura, 35516, Egypt.
[3]Department of clinical pathology, Faculty of Veterinary Medicine, Mansoura University, Mansoura, 35516, Egypt.
4. Department of Animal Medicine, Faculty of Veterinary Medicine, Zagazig University, Egypt.

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***Correspondence to:**
ramy_fouad2001@yahoo.com

ABSTRACT

The present study describes an attempt to determine whether a combination of vaccination and extended antibiotic treatment would eliminate *Staph. aureus* mastitis in lactating dairy cows. Twenty cows infected with *Staph. aureus* mastitis were split into 4 groups; one group (Group A): received 2 injections of *Staph. aureus* bacterin (Lysigin®) with 14 days intervals, the second group (Group B): received 3 injections of Marbofloxacin 10% (Marbocyl® 10%) (by intramuscular route daily for 3 successive days), the third group (Group C): received 2 injections of Lysigin® with 14 days intervals + 3 doses of Marbocyl® 10% (daily for 3 successive days) while the fourth group (Group D): control cows did not receive any treatment. In our trial we found that the *Staph. aureus*-clearance rate in all cows at the level of quarters were 20%, 33.3%, 53.8% and zero %, respectively. Combination of bacterin with extended antibiotic therapy was successful in this trial as results indicated that a combination of vaccination and antibiotic treatment can be successful in eliminating 53.8% % of *Staph. aureus* mastitis.

1. INTRODUCTION

Staph aureus is often considered to be a major mastitis-causing pathogen in dairy herd Fox et al., 1995; Nickerson et al., 1995; Owens et al., 2001; Oliver et al., 2004 and Piepers et al., 2009. Unfortunately, this pathogen has powerful resistance to treatment with antimicrobials during lactation resulted in low clinical cure rates Sears et al., 2003. Antimicrobial failures have been attributed to lack of susceptibility, limited exposure of the bacteria to the antimicrobial and poor immune function due to pathogen characteristics such as the ability to survive inside the host cell, pathological changes and abscesses formation induced in chronic infections Bramley, 1992. Because cows with chronic *Staph. aureus* mastitis is considered as a threat to other cows in the herd, infected cows are generally culled. However, many dairy producers either are unwilling to cull these cows or have so many infected cows that it is impossible and uneconomical to cull them all. Also the increasing public concern with food safety to minimize antibiotic residues in milk, on one hand and the need to reduce somatic cell counts on the other hand .Therefore, it is important to develop novel treatment methods to eliminate intramammary *Staph. aureus* infections.

Clinical trials of extended Marbofloxacin 10% administration have yielded promising results in eliminating intramammary *Staph. aureus* infections. Cure rates vary widely among studies due to several factors influence the success of the treatment, including age of the cow, duration of infection, somatic cell count at the time of treatment, presence of infection in the front quarters, stage of lactation, and strain of *Staph. aureus* (Taponen et al., 2003; Deluyker, 2005). Use of antibiotics must be according to Food & Drug Administration regulations, and appropriate with holding times for meat and milk must be observed.

To resolve the problem of *Staph. aureus* mastitis and to prevent new cases of intra mammary infections, staphylococcal vaccines had to be used.

Most studies used bacterins developed from single strains of *Staph. aureus* to detect whether these bacterins are able to induce long-lasting immunity and whether they are capable of markedly reducing intramammary *Staph. aureus* infections (Tenhagen *et al., 2001).* Immunization with *Staph. aureus* bacterin stimulate strong humoral responses in blood and milk and associated with reduced bacterial cell count and less sever clinical signs after experimental challenge (Prenafeta et al., 2010). Capsular polysaccharides are considered important components for vaccine development because of its capability of inducing strong humoral and cellular responses in a variety of animal species, through the induction of multiple innate and adaptive mediators, cellular processes and the interplay between these elements as antibodies against them, opsonize *Staph. aureus* and enhance neutrophil phagocytosis (Guidry et al., 1991; Guidry et al., 1994; Pearse and Drane, 2004; Sun et al., 2009).

The goal is to enhance the animal's immune status before antibiotic treatment to enhance bacteriologic clearance, as a study by Timms and Sears, 2004 reported a better results with combinations of polyvalent *Staph. aureus* bacterin and intramuscular antibiotic treatment (pirlimycin) than antibiotic alone. In theory at least, vaccine-antibiotic combination may potentially be more effective in the treatment of mastitis in dairy cattle than antibiotics alone.

Therefore, the aim of the study discussed here was to investigate the ability of vaccination and antibiotic treatment to control *Staph. aureus* mastitis in dairy cows.

2. MATERIAL AND METHODS

This study was committed by Faculty of Veterinary Medicine, Mansoura University and Animal Health Research Institute. The main goal of the trial was to identify cows with *Staph. aureus* mastitis. Between 120 lactating cow in dairy farm at Damietta governorates, 20 cows were infected with *Staph. aureus* mastitis (16.6%) and the initial aseptic quarter samples from 20 cows showed that 40 quarters (57.1%) were infected with *Staph. aureus*.

2.1 Animals:

A total of 120 lactating cows from a dairy farm in Damietta Governorate, Egypt, were screened by California mastitis test (CMT) according to **Radostitis *et al.*, 2000** for detection of sub clinical mastitis. Positive quarters for CMT were labeled and duplicate milk samples were collected from positive quarters for bacteriological culture. Each quarter of cows that yielded positive culture results for *Staph. aureus* were identified.

Cows were excluded from the study if *Staph aureus* was not isolated from both duplicate milk samples and at least 1 quarter. All cows with positive *staph. aureus* cultures in duplicate samples of 1 or more quarters were considered infected and were included in the study. Thus, all cows included in the trial had 1 or more quarters that yielded positive results for *Staph. aureus* isolation .

2.2 Experimental protocol:

Twenty Holstein cows with *Staph. aureus* mastitis were identified to be included in the study. Cows were divided into 4 groups; five animals per each group.

Group (A): injected with a polyvalent *Staph. aureus* bacterin (Lysigin®) (2 doses, 2 weeks intervals / 5ml intramuscular).

Group (B): injected with Marbofloxacin (Marbocyl® 10%, Laboratoire veto quinol, lure, France, Batch No. 6C1659E) (2mg/kg in a single daily injection for 3 successive days by intramuscular route).

Group (C): injected with Lysigin® (2 doses, 2 weeks intervals / 5ml intramuscular) + Marbocyl® 10% (2mg/kg in a single daily injection for 3 successive days by intramuscular route).

Group (D): control cows did not receive any treatment.

2.3. Samples:

2.3.1 Collection of milk samples:

During milking process, duplicate milk samples from quarter fore-milk were collected aseptically from all cows at 0, 15 and 30 days of treatment for bacteriological culturing as described by Honkanen-Buzalski, 1995.

2.3.2 Collection of serum samples:

Serum samples from all cows were obtained at 0, 4, 7, 14, 21 and 28 days of treatment for analysis of serum *Staph. aureus* antibody titers. Blood samples were collected by tail vein puncture, samples were placed on ice, processed by centrifugation (10 minutes at 3000rpm), and the serum samples were collected and stored at – 20°C until utilized for determination of total antibodies titres against *Staph. aureus* by enzyme linked immunosorbent assay (ELISA) according to Mclaren et al., 1981.

2.3.3 Whole blood samples:

Whole blood samples (heparinized) from all cows were obtained at 0, 4, 7, 14, 21 and 28 days of treatment for cellular immune response via tail vein puncture. Samples were transported to faculty of veterinary medicine infectious diseases laboratory within two hours for measuring some cellular immune response.

2.4. Bacteriological culture procedures:

Independently, quarter milk samples were streaked on selective media (Baird-Parker) and followed by confirmation of the suspected colonies by colony morphology, hemolytic pattern, and catalase test and tube coagulase-test. Identifying a single colony of *Staph aureus* is enough to define the quarter as infected. Culture procedures were done as described by the National Mastitis Council (Oliver *et al.,* 2004).

2.5. Preparation of Staph suspension:

Staph aureus strain was obtained randomly from one of the mastitic cattle milk samples involved in the experiment flock following the isolation and identification at the infectious diseases lab. Faculty of Veterinary Medicine, Mansoura University. The preparation of the *Staph aureus* suspension was performed according to Saikia *et al.*, 2003, with some modifications. In details, one colony of the *Staph. aureus* was inoculated in nutrient broth (Oxoid) and incubated at 37°C for 24 h, then it was streaked on Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) and incubated at 37°C for 24 h. The colonies were harvested into 10 ml of phosphate buffered saline (ph 7.2) in covered tube, shacked well and vortex for 10 min. For killing the *Staph. aureus*, the tube was incubated in a 56°C water bath for 60 min. It was centrifuged at 1500 rpm for 8 min then washed twice with the same buffered saline. The final concentration (10^8 cells/ml) was adjusted by turbidity measurements at 550 nm (Chapman *et al.*, 2002). The suspension was preserved in refrigerator until used.

2.6. Antibody titre determination:

Serum samples from all treated and control cows were obtained at 0, 4, 7, 14, 21 and 28 days of treatment for determination of total antibodies titres against *Staph. aureus* by enzyme linked immunosorbent assay (ELISA) according to **Mclaren *et al.*, 1981,** the prepared *Staph. aureus* suspension used in coating of the plates.

2.7. Cellular immunological analysis:

2.7.1. Total and differential leukocytic count:

The TLC count and differential leukocytes count were performed manually using improved Neubauer hemocytometer (Feldman *et al.*, 2000).

2.7.2. Phagocytic activity and phagocytic index essays:

Phagocytic activity of the neutrophil leukocytes was performed according to **(**Saikia *et al.*, 2003) with few modifications. One millilitre of heparinized blood of each sample was put in a glass tube in paired with 50 µl of fetal calf serum (Sigma) then added 50 µl of the Staphylococcus suspension. Gently, the samples were mixed and incubated at 37°C for 20 min. From each tube sample, two blood smears were prepared, fixed with methanol and Geimsa stained.

2.7.3. Phagocytic activity and phagocytic index:

Phagocytic activity was the ratio between the number of phagocytosis neutrophils and the number of all assessed neutrophils. The phagocytic index was calculated as the ingested particles average number per phagocytosis neutrophils (Berger and Slapničková, 2003).

3**. Statistical analysis**

Analysis of variance (ANOVA) with LSD Test was used for analyzing data using SPSS 17 program for windows. Results were expressed as mean ± SE. Two groups were different significantly when the P value was statistically lower than 0.05.

4. RESULTS

4.1. Bacteriological examination:

At the end of the trial, bacteriological examination of milk samples collected at the 30 days of treatment revealed that the combination of vaccination and intramuscular administration of Marbofloxacin have a significantly higher bacteriological clearance rate (7/13) quarters [53.8%]), compared with the control group (0/5 [0%]). In comparison to treated group with Marbofloxacin (4/12 [33.3%]), and with the cows received Lysigin® only (2/10 [20%]). **4.2. Antibody response:**

4.2.1. Humoral immune response:

Before treatment, there were no significant differences between OD values of anti-staphylococcal antibodies in the 4 groups. After immunization, all the cows in the vaccinated group were found to be positive, with increased antibody levels. In non-immunized group, the individual antibody levels fluctuated but few of the originally antibody-negative cows had become antibody-positive. Table (2) and Fig. (2). Values with different superscripts within column differ significantly (P<0.05)

Table (1): *Staph aureus*-clearance rate in quarters of the treated cows at the end of the trial (at the 30 day from the beginning of the treatment) .

Group	No. of quarters with *Staph. aureus* before treatment	No. of quarters with negative isolation of *Staph. aureus* after treatment	Clearance rate
Group(A)	**10**	**2**	**%20**
Group(B)	**12**	**4**	**%33.3**
Group(C)	**13**	**7**	**% 53.8**

Group(D)	5	zero	% 0.0

Table (2): The mean antibody levels of *Staph. aureus* in the serum of all groups:

Group	Antibody levels (Days after treatment)					
	0Day	4 Days	7 Days	14 Days	21 Days	28 Days
Group (A)	0.13	0.19	0.22	0.29	0.41	0.38
Group (B)	0.14	0.17	0.19	0.20	0.21	0.22
Group (C)	0.23	0.29	0.31	0.43	0.49	0.52
Group (D)	0.14	0.15	0.17	0.18	0.19	0.18

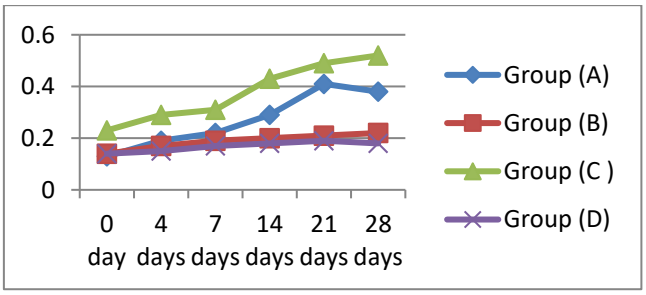

Figure (2): The mean antibody levels of *Staph aureus* in the serum of all groups.

4.2.2. Cellular immune response:

Before treatment, the blood lymphocyte proliferative responses were nearly similar in all groups. After immunization lymphocyte proliferative responses were varied between immunized groups. Lymphocyte responses were significantly higher in Group (C) than other groups.

Table (3): Leukogram (Mean ± S.E) of mastitic cows with *Staph aureus* and immunized with *Staph aureus* vaccine and Marbofloxacin

Periods	Groups	TLC /10^3µL	Lymphocyte /10^3µL	Neutrophil /10^3µL	Monocyte /10^3µL	Eosinophil /10^3µL
0 day	A	8.27±0.45	4.23±0.24	3.62±0.52	0.21±0.02	0.17±0.04
	B	8.32 ±0.42	4.91±0.35	3.00±0.31	0.21±0.02	0.17±0.02
	C	8.68±0.20	5.01±0.18	3.29±0.15	0.22±0.05	0.14±0.02
	D	8.26±0.30	4.25±0.25	3.65±0.20	0.14±0.02	0.20±0.0
4th day	A	10.62 ± 1.42a	6.31 ± 1.44	4.17b ± 0.45b	0.14 ± 0.06	0.02 ± 0.02a
	B	7.75 ± 0.35b	4.27 ± 0.38	3.08a ± 0.26a	0.26 ± 0.06	0.09 ± 0.03b
	C	10.21± 0.60ab	7.07 ± 0.56	2.77a ± 0.29a	0.29 ± 0.02	0.07 ± 0.02a
	D	7.55±0.35b	4.30±0.30	3.07±0.25a	0.16±0.02	0.02±0.0a
7th day	A	9.22 ± 0.42ab	5.39 ± 0.50	3.43 ± 0.56	0.19 ± 0.01ab	0.18 ± 0.04b
	B	8.14 ± 0.41b	4.44 ± 0.78	3.49 ± 0.70	0.14 ± 0.05b	0.06 ± 0.03ab
	C	10.82 ± 0.72a	5.38 ± 0.30	5.04 ± 0.58	0.30 ± 0.04a	0.11 ± 0.01a
	D	7.27±0.40b	4.0±0.46	2.95±0.31	0.16±0.02b	0.12±0.0a
14th day	A	9.90 ± 1.15ab	6.23 ± 0.88b	3.29 ± 0.31	0.23 ± 0.02	0.12 ± 0.01b
	B	8.27 ± 0.45b	4.23 ± 0.24b	3.62 ± 0.52	0.22 ± 0.05	0.16 ± 0.02bc
	C	12.59 ± 1.37a	8.84 ± 0.84a	3.23 ± 0.45	0.18 ± 0.09	0.33 ± 0.09a
	D	7.25±0.50b	4.09±0.45	2.80±0.30	0.17±0.02	0.20±0.0c
21st day	A	9.90 ± 1.15ab	6.23 ± 0.88b	3.29 ± 0.31	0.23 ± 0.02	0.12 ± 0.01b
	B	8.87 ± 0.40b	5.18 ± 0.34bc	3.28 ± 0.11	0.22 ± 0.05	0.16 ± 0.02bc
	C	12.59 ± 1.37a	8.84 ± 0.84a	3.23 ± 0.45	0.18 ± 0.09	0.33 ± 0.09a
	D	7.45±0.60b	3.95±0.52bc	3.13±.030	0.17±0.02	0.20±0.0c
28th day	A	10.40±.81a	6.65±.81a	3.33±0.45	0.25±0.02	0.17±0.02
	B	8.32 ± 0.45b	4.26 ± 0.24b	3.68 ± 0.52	0.21 ± 0.03	0.17 ± 0.04
	C	13.4± 0.6a	9.55±0.72a	3.35±0.30	0.18±0.02	0.22±0.01
	D	7.73±0.84b	4.20±0.50b	3.15±0.32	0.18±0.03	0.20±0.0

Means with the same letter in the same column (in the same period) are not significantly different at P < 0.05.

Table (4): cellular immunological parameters (Mean ± S.E) of mastitic cows with *Staph aureus* and immunized with *Staph aureus* vaccine and Marbofloxacin.

Days / Groups	0	4	7	14	21	28
phagocytic activity ratio						
A	28.45 ± 5.83	52.19 ± 5.74b	55.84± 5.0b	29.87±6.25ab	29.87± 6.25	29.24±1.02c
B	30.24 ± 7.54	27.49 ± 1.82a	19.24± 3.31c	20.32± 2.68b	17.58± 1.87c	16.26±1.56b
C	30.41 ± 5.93	22.66 ± 1.65a	71.90± 3.02a	66.08± 9.81a	66.08± 9.81a	72.90± 3.0a
D	25 ±1.5	19.20±2.60a	20.23±2.60c	19.33±5.65b	18.23±3.32c	17.50±1.60b
phagocytic index						
A	1.64 ± 0.34	2.49 ± 0.21b	3.11± 0.37b	2.49± 0.31a	2.49± 0.31a	3.50±0.30
B	1.81 ± 0.21	1.56 ± 0.30a	1.13± 0.07c	1.09± 0.05b	1.13± 0.07b	1.10±0.25
C	1.75 ± 0.39	1.50 ± 0.27a	4.64± 0.35a	3.75± 0.21a	2.75± 0.21a	3.90±0.30
D	1.72±0.24	1.65±0.25a	1.55±0.33c	1.13±0.17b	1.10±0.25b	1.09±0.05

Group (A): infected and received *Staph. aureus* vaccine. Group (B): infected received Marbofloxacin 10%. Group (C): infected received *Staph aureus* vaccine + Marbofloxacin. Group (D):infected control. Means with the same letter in the same column (in the same period) are not significantly different at P < 0.05.

5. DISCUSSION

Cattle included in this study were considered infected with *Staph aureus* on the basis of results of bacteriological culture before the start of the trial, and bacteriological clearance was defined as negative culture results for *Staph aureus* after treatment. Quarters evaluated via pre-treatment cultures Sol *et al.*, 1997.

The present study showed that, combination of vaccine (*Staph. aureus* bacterin) with extended antibiotic (Marbofloxacin 10%) improve the overall efficacy of the treatment protocol used in the present study, compared with extended intramuscular antibiotic alone, as cattle that received antibiotic and vaccination had a significantly higher cure rate of quarters (53.8%) than those that received only antibiotic therapy only (33.3%). It is likely that the use of *Staph aureus* bacterin to treat intramammary *Staph. aureus* infections provides higher efficacy (20%) than control (0%).

Bacteriologic clearance rates in this study were lower than have been reported by Tanveer Ahmad 2009, who found that the bacteriologic clearance rate (83%) at day 28 post treatment was significantly higher in *Staph aureus* infected quarters treated with combination of bivalent bacterin-toxoid and antibiotic therapy than that of antibiotics alone (53%) as well as bivalent bacterin-toxoid alone (39%). In *Staph. aureus* infected quarters treated with antibiotics alone, clinical cure rate (53%) was significantly higher than corresponding outcome (39%) in quarters treated with bivalent bacterin-toxoid alone .

In another study made by Czernomysy-Furowicz *et al.*, 2014, to compare the effectiveness of cefuroxime, herd-specific autovaccine, and cefuroxime/herd-specific autovaccine treatments in eliminating *Staph aureus* from the milk of 45 cows with subclinical mastitis, it was revealed that after day 35 of the treatment *Staph. aureus* was not detected in the milk of 40% of the cows treated with antibiotic, 60% of the cows treated with the autovaccine, and 100% of the cows that underwent combined therapy.

The influence of a combination of vaccination and extended antimicrobial treatment in elimination of intramammary *Staph aureus* infections in lactating dairy cows were evaluated by Smith *et al.*, 2006. These authors demonstrated a significant reduction in mastitis cases in vaccinated and antibacterial-treated cows, providing evidence that vaccine and an antibiotic can have synergistic action, which agree with our study.

In another study made by Leitner *et al.*, 2004 a group of cows with *Staph. aureus* infections was vaccinated with a polyvalent bacterin, whereas another group received only antimicrobial treatment; at the end of the trial 30% of the vaccinated cows were considered cured, compared with only 6% of the control cows.

Vaccine administration enables antigen presentation through the antigen presenting cells to Th1 lymphocytes, which in turn stimulates B cells to produce specific IgG2. The antibodies appear more quickly after re-vaccination, remain longer, and consist mainly of IgG2 according to Nansen, 1972; Caffin *et al.*, 1988.

Moreover, in our study *Staph aureus* bacterin administered during *Staph aureus* mastitis resulted in increasing secondary immune response which resulted in a short-time synthesis of large amounts of highly specific antibodies. Also polymorphnuclear neutrophils play the most important role in mammary gland protection against

microorganisms flowing into the gland coming from blood and settling in the gland tissue Paape et al., 2002; Rainard et al., 2006. The increase in phagocytic activity observed after vaccine administration kills staphylococci located in and outside neutrophils Heyneman et al., 1990; Kasprowicz et al., 1994. However, lack of full effectiveness in elimination of *Staph aureus* after vaccination can be a result of defensive strategies of *Staph. aureus*.

As suggested by Smith *et al.*, 2006, the antibiotic eliminates extracellular bacteria, whereas the vaccine stimulates synthesis of anti-*Staph aureus* immunoglobulin G2 and enhances phagocytic activity, leading to digestion of engulfed (intracellular) bacteria.

IgG is the principal immunoglobulin of the mammary gland immune system, responsible for promoting neutrophils phagocytosis Miller *et al.*, 1988; Avery *et al.*, 1991, therefore, high levels of antibodies in the blood stimulate the transfer of antibodies to the milk, to combat with infections.

6. CONCLUSION

Results of this study showed that the combined use of *Staph. aureus* vaccine and extended intramuscular antibacterial therapy have a relatively high probability of curing and eliminating *Staph. aureus* mastitis in dairy cows through enhancing the animal's immune status and stimulating strong humoral immune responses in blood and milk. As raising antibodies leads to increasing opsonic capacity with antibacterial treatment to improve bacteriological clearance of infection.

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