

EFFECT OF MONTANIDE ADJUVANTED *Staphylococcus aureus* BACTERIN-TOXIOD ON PREVALENCE AND INCIDENCE OF MASTITIS IN COWS

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The current study was conducted to investigate the prevalence and incidence of mastitis associated with Montanide adjuvanted *Staphylococcus aureus* bacterin toxiod in dairy cows in Faisalabad (Punjab, Pakistan). A total of 60 mastitis free dairy cows, in first and second month of lactation, were selected on the basis of results determined by California mastitis test and Surf field mastitis test. The animals were randomly divided into three groups (C1, C2, and C3) each containing twenty cows. The vaccine was administered twice with four weeks interval at the rate of 5 ml intramuscularly in the neck region in all the animals of group C1 and C2. Levamisole HCl (Nilverm®, ICI, Pakistan) @ 2.5 mg per kg weight was given orally to animals of group C2 after first and second injections of vaccine, while the animals in group C3 were kept as control (non-vaccinated and non-medicated with levamisole HCl). Prevalence and incidence rates were determined at 1st, 30th, 60th, 120th and 180th days post-vaccination. The results showed that maximum numbers of quarters were found positive in group C3 with maximum cumulative prevalence (27.5%) while minimum (11.25%) was recorded in group C2 followed by vaccinated group (13.75 %). There was no significant variation in cumulative incidence between the vaccinated and vaccinated plus levamisole HCl group.

Keywords: Montanide, *Staphylococcus aureus*, incidence, mastitis, cows, inflammation

INTRODUCTION

Mastitis, the inflammation of mammary glands, is a major production limiting disease of dairy animals all over the globe. It not only reduces milk quantity but also impairs quality thus adversely affecting milk production economics (DeGraves and Fetrow, 1993; Sordillo *et al.*, 1997). World-wide losses due to mastitis have been estimated as 35 billion dollars annually. In some instances being zoonotic in nature, it also poses public health risk (Srinivasan and Singh, 1988). As in many countries, mastitis is one of the most important health problem of dairy animals in Pakistan (Ajmal, 1990). Mastitis can be caused by a wide variety of microorganisms but *S. aureus* is the most common etiological agent (Philpot and Nickerson, 1991; Giraudo *et al.*, 1997). It is associated with chronic and sub-clinical mastitis that may sometimes appear in clinical form, in spite of reasonable improvements in farm management and hygiene, milking techniques and treatment strategies; hence it emerged as a major problem of world dairy industry (Watson, 1989; Leslie and Dchukken, 1993; Eckersal *et al.*, 2001).

The control of mastitis becomes more difficult in countries like Pakistan where there is no mastitis control program in practice. Standard mastitis control practices recommended by National Mastitis Council, Inc., USA (Nickerson, 1994) are difficult to adopt because of small herd size, farmer's illiteracy and lack

of milk quality premium. In the absence of any mastitis control program and presence of high antibiotic resistance, vaccination against some of the most prevalent mastitis pathogens seems better choice to curtail the losses caused by mastitis. Ideally, a mastitis vaccine should be able to establish an inflammatory response in order to get rid of infection and at the same time should prevent occurrence of a new infection. Many workers have reported the role of vaccination in the control of mastitis (Cullor, 1991; Watson *et al.*, 1993; Giraudo *et al.*, 1997; Yancey, 1999; Leigh, 2000). Montanides are adjuvants developed by rendering water in oil emulsions. The mastitis vaccine containing Montanide induces significantly higher immunity to α and β toxins in sheep (Tollersrud *et al.*, 2001). Montanide® (Seppic, France) is reported to elicit better and quicker immune response (Athar, 2007) suggested co-administration of immune response modifiers like levamisole HCl with mastitis vaccines. The immunomodulatory properties of levamisole HCl are well established both in medical and veterinary research (Ishikawa *et al.*, 1982). The levamisole HCl therapy reduced the incidence of mastitis from 9.6% to 3.7%. However, mechanism of the role of levamisole HCl immunopotentiator in mastitis vaccine has not been investigated thus far. Thus, keeping in view the importance of *S. aureus* mastitis in Pakistan, the present study was designed to evaluate the effect of Montanide adjuvanted *S. aureus*

bacterin toxoid with or without levamisole HCl on prevalence and incidence of *S. aureus* mastitis in dairy cows.

MATERIALS AND METHODS

Procurement of isolates

The standard *S. aureus* culture for vaccine preparation was procured from Mastitis Research Lab., Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan (Butt, 2006) and was re-characterized for future use.

Re-characterization of selected isolates

The glycerol preserved culture was propagated on blood agar plates followed by Staph 110 medium. Single colony of *Staphylococcus* was transferred onto the next culture plates to obtain pure culture to study morphological and cultural characteristics as described by National Mastitis Council, Inc., USA (1990). The standard suspension of 0.5 MacFarland was prepared from the pure culture and was further processed through API STAPH-Trac (BioMerieux, France) and numerical data were collected. Pure growth was maintained on trypticase soya broth containing 20% glycerol at -70°C for further use (Muhammad, 1992).

Preparation of plain bacterin

The selected vaccinal isolates of *S. aureus* were grown at 37°C for 48 hours on an orbital shaker set at 60 rpm under aerobic conditions. The suspension was confirmed for culture purity and expression of pseudo capsule followed by inactivation by addition of formalin (0.4% V/V), centrifuged at 6000xg for 1 hour at 4°C and resuspended in PBS (pH 7.2). The suspension was then preserved at 4°C until required. Bacterial concentration was then adjusted to 1x10⁸ CFU per ml sodium spectrophotometrically (Hirsch and Strauss, 1964). Sodium azide, thimerosal and formalin were added at final concentration of 0.001% (W/V), 0.001% (W/V) and 0.4% (V/V) respectively.

Preparation of Montanide (Seppic, France) adjuvanted *S. aureus* bacterin-toxoid

Montanide adjuvanted bacterin-toxoid was prepared by adding Montanide[®] ISA 206 (Seppic, France) 50/50 (W/W) ratio to a suspension of plain bacterin and alpha and beta toxoid. The beaker containing oil phase was fitted to a homogenizer and stirred at 200 rpm. The antigen in aqueous phase was then added slowly in 30 seconds. Stirring was increased to 2000 rpm for 10 minutes. The 5ml of Montanide adjuvanted bacterin-toxoid finally contained 1x10⁸ cells of *S. aureus*, 1.5ml of toxin extract, 0.02ml formalin, 0.00005 g of

thimerosal, 0.00005 g of sodium azide and Montanide[®] ISA 206 to make a total volume of 5ml.

Prevalence of mastitis

Prevalence of mastitis was determined on the basis of California mastitis test (Schalm *et al.*, 1971) and Surf field mastitis test (Muhammad *et al.*, 1995) and microbiological examination of four quarters milk samples positive in both above mentioned test at 1st, 30th, 60th, 120th and 180th day post vaccination (National Mastitis Council, Inc, USA, 1990). Cumulative prevalence was also calculated using the total number of mastitic quarters in each experimental group.

Cumulative incidence

Incidence rate of mastitis was calculated on the basis of results of California mastitis test (Schalm *et al.*, 1971) and Surf field mastitis test (Muhammad *et al.*, 1995). The incidence rate was determined for the period intervening between day 1 and day 30, day 31 and day 60, day 61 and day 120 and day 121 and day 180. Cumulative incidence for the period between day zero and day 180 was also calculated to compare between the experimental groups.

RESULTS AND DISCUSSION

Confirmation of *S. aureus* isolates

The standard *S. aureus* culture was confirmed through morphological and cultural characteristics on blood agar plates and Staph 110 medium. This was found as typical gram positive cocci. On blood agar plates, the colonies were cream colored with smooth surface, rounded in shape having entire edges. They were surrounded by a partial and complete haemolytic pattern being an indicator of α and β haemolytic character. The typical colony with characteristic golden color, smooth raised and entire edges were found visible on the surface of Staph 110 medium after 48 hours of incubation. The selected isolate of *S. aureus* was biotyped again on API Staph Trac, which showed identification number allotted to culture as 6736153 and it was identified as *Staphylococcus aureus* with 98% confirmation.

Quarter-based prevalence

Number of quarters detected positive for mastitis on basis of Surf field mastitis test (SFMT)/California mastitis test (CMT) at different days post-vaccination are presented in Table 1. The maximum numbers of quarters were found positive in the control group that received neither vaccine nor levamisole HCl followed by group that received vaccine (Montanide adjuvanted *S. aureus* bacterin toxoid) but no levamisole HCl.

Minimum number of quarters that showed mastitis at different days post vaccination recorded in the group that received vaccination with oral levamisole HCl administration. The point prevalence (%) given in Table 2, indicated that minimum cumulative prevalence (11.25%) in group C2 that was levamisole HCl modulates vaccine group followed by vaccinated group (13.75%). Maximum cumulative prevalence was noted in control group (27.5%).

Incidence rate at day 60 in control group was the highest throughout the experimental period. The control group showed the highest cumulative incidence rate during the study period while the vaccinated group showed an equal cumulative incidence of 7.5% regardless of modulates of levamisole. In the vaccinated group, the highest incidence rate was noted (2.5%) at any sampling day, while it rises to the maximum of 6.25% in the control group. The results

Table 1. Number of quarters found positive based on detection by CMT/SFMT in vaccinated and non-vaccinated cows at different days post-vaccination

Groups of cows	Day 0	Day 30	Day 60	Day 120	Day 180
Vaccine	0	2	3	3	3
Vaccine+Levamisole	0	1	3	2	3
Control	0	4	7	6	5

Table 2. Quarter-based point prevalence (%) of mastitis in vaccinated, vaccinated plus levamisole medicated and control cows at different days post-vaccination

Groups of cows	Quarter-based point prevalence (%) of mastitis at different post-vaccination days					Cumulative prevalence (%)
	Day 0	Day 30	Day 60	Day 120	Day 180	
C1	0	2.5	3.75	3.75	3.75	13.75
C2	0	1.25	3.75	2.5	3.75	11.25
C3	0	5.0	8.75	7.5	6.25	27.5

Incidence of mastitis

Number of new quarters detected on basis of Surf field mastitis test/California mastitis test on different days post-vaccination in three experimental groups of cows (C1, C2 and C3) has been showed in Table 3, while quarters-based incidence rate is showed in Table 4.

showed that no significant difference was found in group C1 and C2. Quarter-based cumulative incidence indicated that modulating with levamisole HCl along with vaccine (Montanide adjuvanted *S. aureus* bacterin toxiod) did not have any effect on the quarter-based incidence.

Table 3. Number of new quarters found mastitic on basis of SFMT/CMT on different days post-vaccination in vaccinated, vaccinated plus levamisole medicated and control cows

Groups of cows	New quarters found positive for mastitis at different post-vaccination days				
	Day 0	Day 30	Day 60	Day 120	Day 180
Vaccine	0	1	2	1	2
Vaccine+Levamisole	0	1	2	1	2
Control	0	4	5	3	3

Table 4. Quarter-based incidence rate (%) in vaccinated, vaccinated plus levamisole medicated and control cows at different days post-vaccination

Groups of cows	Quarter-based incidence rate (%) at different days post-vaccination					Cumulative incidence (%)
	Day 0	Day 30	Day 60	Day 120	Day180	
C1	0	1.25	2.5	1.25	2.5	7.5
C2	0	1.25	2.5	1.25	2.5	7.5
C3	0	5.0	6.25	3.75	3.75	18.75

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. If vaccine is successful in bringing down prevalence and incidence of *S. aureus* mastitis, then it would significantly curtail the economic losses due to mastitis. In the present study, Montanide® adjuvanted *Staphylococcus aureus* bacterin toxoid vaccine performed well in terms of reducing the cumulative point prevalence and cumulative incidence at different sampling intervals. The reduction in number of clinical mastitis cases has also been reported by Tollersrud (2002). Norcross and Kenny (1994) reported fewer new infections in vaccinated cows as compared to control. Works of Nordhaug *et al.* (1994); Nickerson *et al.* (1997) and Giraudo *et al.* (1997) also reported reduction in point prevalence and incidence rate of mastitis in vaccinated animals. The minimum point prevalence (11.25%) was observed in vaccinated plus levamisole adjuncted in the present study. The study of Ishikawa *et al.* (1982) also supported the use of levamisole in mastitis control indicating a cure rate of 60% when used orally in 20 sub-clinically mastitic cows on day 28 post-treatment.

CONCLUSION

Based on the findings of the present study, it was concluded that vaccine with oral administration of Levamisole HCl @ 2.5 mg per kg weight gave better results on prevalence and incidence of *S. aureus* mastitis in dairy cows.

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