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Evaluation of cell mediated immune response in bovine mastitis causing *Staphylococcus aureus* biofilm vaccine

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Abstract: *The effect of bovine mastitis causing Staphylococcus aureus (S.aureus) biofilm (BF) vaccine on lymphocyte subpopulations with respect to CD4 and CD8 cells was evaluated in lactating cows by flow cytometry. In all, eighteen cattle in early lactation which were free from mastitis were subjected to trials. Bentonite clay based S.aureus BF and free cell (FC) vaccines were administered at 0, 30 and 60 days using Freund's Incomplete Adjuvant (FIA) in the first shot. In flow cytometric analysis, S.aureus BF vaccinated group showed a significant enhancement in CD4 and CD8 cells. The percentage of CD4 and CD8 increased significantly in the S.aureus BF vaccinated groups than in the control group and furthermore, in comparison with S.aureus FC vaccine, the percentage of CD4 and CD8 cell population found to be marginally increased on days 60 and 90 and significantly increased on day 120 post vaccination.*

Keywords: *Mastitis; Bovine; Staphylococcus aureus; Biofilm vaccine; Cell mediated immune response*

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I. Introduction

Bovine mastitis is an infectious disease with a major economic influence on dairy production. *Staphylococcus aureus* is one of the main causative agents of bovine mastitis. *S.aureus* intramammary infections (IMI) tend to become persistent and chronic, even in animals placed under antibiotic treatment (Gomez *et al.,* 2002). The prevention of mastitis is better than its control. In view of this, number of studies in the last 40 years have aimed to develop a worldwide efficacious vaccine against mastitis caused by *S.aureus*. However, a well-studied commercially available vaccine being able to reduce the incidence of new IMIinfection or eliminate chronic mastitis is not available.The reasons include an incomplete knowledge of virulence factors, the interference of milk components with antibody function and immunocytes and the best delivery system including use of adjuvants (Leitner*et al.,* 2003a). It has been established that, IMI involving *S. aureus* and *E. coli* are associated with BF formation (Baselga*et al.,* 1993; Cucarella *et al*., 2004; Fox *et al.,* 2005 and Melchior *et al*., 2006). Although, therapeutic agents may reverse the symptoms caused by free cells (FC) released from the BF, they fail to kill the BF. For this reason, BF infections typically show recurring signs after antibiotic therapy. However, vaccine still remains a promising tool to control *S.aureus* IMI, but their efficacy under field conditions in different environments should be demonstrated. The present study was designed to evaluate T cell proliferative response in cattle by analyzing lymphocyte subpopulation with reference to CD4 and CD8 cells in mastitis causing *S.aureus* BF vaccinated and FC vaccinated cattle by Flow cytometry.

II. Material and Methods

Preparation of vaccines

Two types of vaccines were prepared; biofilm and free cell or planktonic vaccines using *S.aureus* SA16. For preparation of biofilm vaccine,three-day-old BF cells were grown in 0.32 percent TSB, incorporated with 0.3 percent bentonite clay were harvested by discarding the supernatant media to remove any FC. Bentonite clay with BF growth were adjusted to a final concentration of 10^9cfu / ml with bovine serum albumin (BSA) after counting number of viable cells. The growth was inactivated with 0.1percentformalin at RT for 24 hrs and stored at 4°C until use. For preparation of free cell vaccine, the culture was grown in 3.0 percent Tryptic Soya Broth (TSB) for 8 hrs at 37°C and centrifuged at 4,000 rpm at 4°C for 10 min. The pellet was washed

thrice and finally re-suspended in phosphate buffered saline (PBS) to contain 10^9cfu/ml after counting number of viable cells. The pellet was inactivated with 0.1 percent formalin at room temperature for 24 hrs and stored at 4°C until use. For sterility test, the vaccine was inoculated onto Nutrient agar, Mannitol salt agar, Blood agar, Brain heart infusion agar and Robertson bullock heart medium in duplicates and incubated at 37°C under aerobic and anaerobic conditions and examined periodically for any bacterial growth up to seven days.

Animals and Immunization Protocol

Eighteen cattle in early lactation which were free from mastitis were subjected to immunization studies. Animals were grouped into three groups during the experiment. The approval of the Institutional Animal Ethics Committee was obtained. Of these 18, six lactating cows were maintained for each of the two *S.aureus* SA16 biofilm and free cell vaccines. Remaining six were controls. Prevaccinal sera and milk were collected from all the cattle.Each of the six lactating cows were immunized subcutaneously with BF and FC vaccines of *S.aureus* SA16. The first dose in both the cases comprised of 5 ml suspension containing $4X10^9$ cells and 1ml of FIA. The subsequent two boosters were administered on days 30 and 60, which contained only $4X10^9$ cells in 5 ml without FIA.

Sampling of Cows

Blood samples were collected on day 0 and then at an interval of every month up to end of the study. The blood samples from all the animals were subjected to Flow cytometry to study cell mediated immune responses.

Optimization of anti-bovine CD4 and CD8 antibodies

An end point titration was carried out to determine the optimum concentration of anti-bovine CD4 and CD8 monoclonal antibodies to be used to bind lymphocytes.Monoclonal anti-bovine CD4 mouse antibody tagged with fluorescein isothiocyanate (Fluorochrome) and anti-bovine CD8 mouse antibody tagged with phycoerythrin (Fluorochrome) were procured from M/s Serotech, USA.It was performed by using the different concentrations 2.5, 5.0 and 7.5 µl of the anti-bovine CD4 and CD8 monoclonal antibodies (1µg/µl) to bind to the lymphocytes.

Protocol for measurement of CD4 and CD8 cells by Flow cytometry

Whole blood was collected using heparinized vaccutainers and 0.2 ml of blood was taken in 5 ml round bottom Fluorescence Activated Cell Sorting (FACS) tubes. Anti-bovine CD4 and CD8 mouse monoclonal antibodies were added (5 µl at concentration of 1µg/ µl) and incubated on ice for 30 min, with intermittent mixing.Cells were washed with 2 ml of phosphate buffered saline - azide (PBS-azide) 1 percent bovine serum albumin (BSA), the supernatant was discarded after centrifuging at 3,000 rpm for 10 min. Blood suspension was mixed with the erythrolyse solution and incubated for 10 min. The supernatant was discarded by centrifuging at 3000 rpm for 10 min. Cells were re-suspended by adding 0.5 ml of 2 percent para formaldehyde (PFA) per tube. Finally, the tubes were taken to FACS machine for cell differentiation or acquisition.

Statistical Analysis

Statistical analysis will made to compare the cell mediated immune response generated in all the three groups *viz.* control *S.aureus* BF and FC vaccinated groups by using two-way ANOVA based on the percentage of lymphocyte subpopulation with reference to CD4 and CD8 cells in whole blood samples collected at day 0, 30, 60, 90, 120 and 150.

III. Results

In present study the immunological responses were assessed by evaluating T cell proliferative response in cattle by analyzing lymphocyte subpopulation with reference to CD4 and CD8 cells.

Flow cytometric analysis revealed a significant difference ($P<0.01$) in the percentage of CD4 cells between *S.aureus* BF vaccinated group and control group on 30^{th} and 60^{th} day and highly significant on 90^{th} and 120^{th} day ($P<0.001$). Whereas, there was a marginal increase in CD4 cells on day 30^{th}, 60^{th} and 90^{th} and significant increase ($P<0.01$) on day 120^{th} in the BF vaccinated group compared to FC vaccinated group. Increasing trend of CD4 cells appeared to continue beyond 120 days also**.** Whereas, FC vaccinated group indicated the increase in percentage of CD4 cells up to the 90^{th} day (11.2 percent) beyond which it continued to reduce. Control group during the period of study showed plateau which ranged from 6.9 to 7.3 percent of CD4 cells (Fig. 2). The difference in value between *S.aureus* BF vaccinated and control group for CD8 cells was also significant on 60^{th} day ($P<0.01$) and highly significant on 90^{th} and 120^{th} day respectively ($P<0.001$). There was a marginal increase in CD8 cells on days 30^{th}, 60^{th} and 90^{th} and significant increase ($P<0.05$) on day 120 in BF vaccinated group compared to FC vaccinated group. Increasing trend of CD8 cells appeared to continue beyond

120 days also. Whereas, FC vaccinated group indicated the increase in percentage of CD8 cells up to day 90th (6.4 percent), beyond which it reduced unlike BF vaccinated group. Control group during the period of study showed plateau which ranged from 3.4 to 4.4 percent of CD8 cells (Fig. 3).

Thus in the present work, vaccination based on the *S.aureus* BF vaccine showed a significant enhancement of CD4 and CD8 cells in the circulatory system which on further migration to mammary gland could play a major role in conferring protection against the mastitis causing pathogens (Table 1. and Fig. 1.).

Fig. 1: Flow cytometry analysis of CD4 and CD8 lymphocyte population in control, *S.aureus* BF and FC vaccinated groups on days 0, 30, 60, 90 and 120.

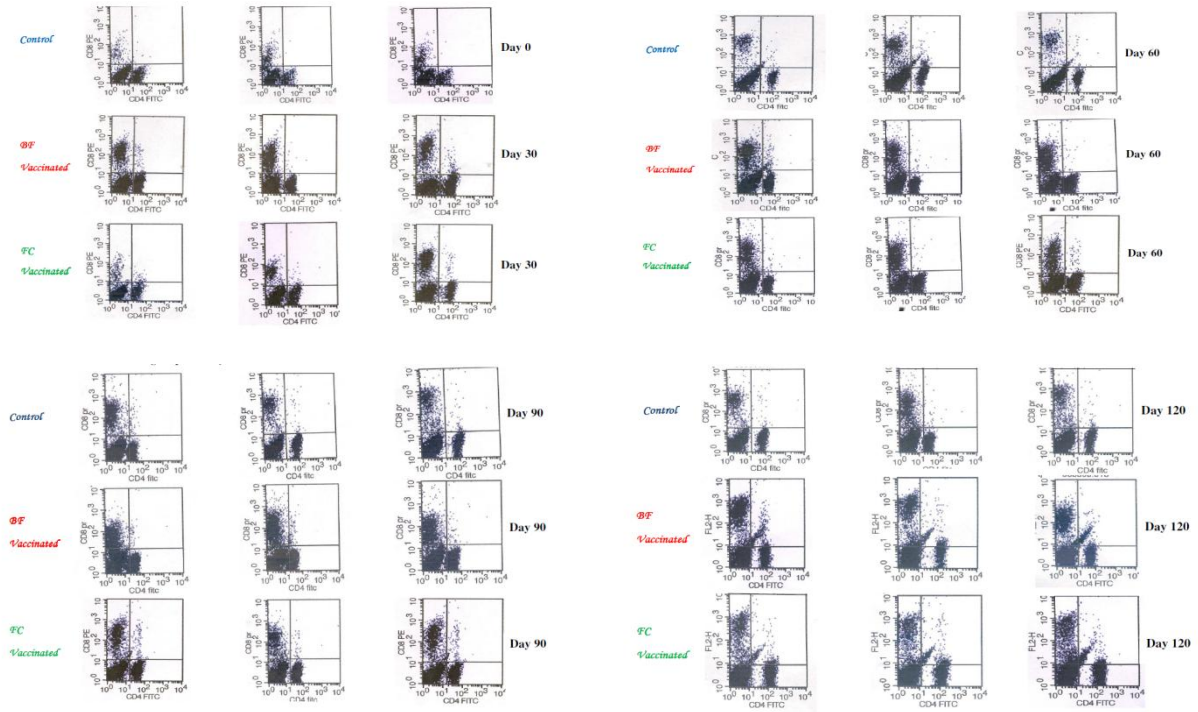

Table 1: Flow cytometric analysis of CD4 and CD8 lymphocyte population from control, *S.aureus* BF and FC vaccinated groups (Mean percentage to total lymphocyte population)

Days blood collected	Control group		*S.aureus* Biofilm vaccinated group		*S.aureus* Free cell vaccinated group	
	CD4	CD8	CD4	CD8	CD4	CD8
Prevaccine 0 days	5.1-9.0 (6.9)	2.2-4.5 (3.4)	5.1-9.0 (6.9)	2.2-4.5 (3.4)	5.1-9.0 (6.9)	2.2-4.5 (3.4)
30 days after vaccine	5.3-9.3 (6.5)	2-4.9 (3.7)	10.2-12.7 (11.8)	4.2-8.9 (5.7)	6.7-10.9 (8.6)	4.2-7.0 (5.2)
60 days after vaccine	6.5-9.2 (7.5)	3.5-5.3 (4.1)	10.4-14.1 (12.1)	6.5-7.0 (6.6)	8.6-12.0 (10.1)	4.7-7.3 (5.8)
90 days after vaccine	6.9-9.1 (7.6)	3.0-6.0 (4.1)	12.4-14 (13.4)	6.0-12 (9.8)	9.8-13.3 (11.2)	5.3-8.7 (6.4)
120 days after vaccine	6.8-8.3 (7.3)	3-5.3 (4.4)	13-16.5 (14.4)	7.3-12.3 (10.2)	9.8-10.5 (10.1)	6.1-6.9 (6.4)

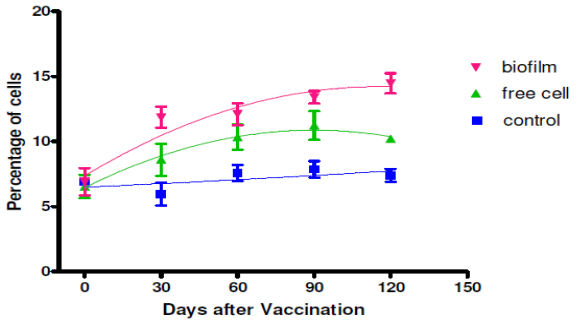

Fig.2: Flowcytometric analysis of CD4 lymphocyte population from control, *S.aureus* BF and FC vaccinated groups (Mean percentage to total lymphocyte population).

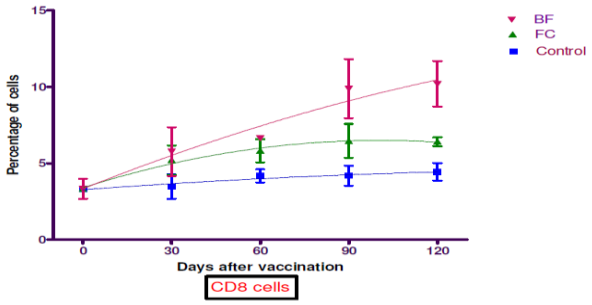

Fig.3: Flowcytometric analysis of CD8 lymphocyte population from control, *S.aureus* BF and FC vaccinated groups (Mean percentage to total lymphocyte population).

IV. Discussion

Bovine mastitis is the most important infectious disease of dairy cows that affects both the quality and quantity of milk (Giraudo*et al.,* 1997). *Staphylococcus aureus* is the most important etiological agent of bovine mastitis (Giraudo*et al.,* 1997) which frequently become chronic, associated with the ability of the bacteria to produce biofilm and also recurrent infections are often attributable to biofilm growth of bacteria (Cucarella*et al.,* 2004 and Melchior *et al.,* 2006). In view of an effective control of mastitis, immunization against mastitis has been a goal of researchers for many years and vaccination against mastitis pathogens is practiced in some dairy farms, especially in western countries. Research on mastitis vaccines has been conducted for at least 35 years. The rationale of our current research is that a deeper knowledge on mammary gland immune responses leading to the eradication of intracellular *S.aureus* is required for the rational design of a vaccine for protecting ruminant hosts from staphylococcal IMI. Generation of an effective, specific immunity involves both antigen presenting cells and lymphocytes. Lymphocytes are the only cells of the immune system that recognize the antigens through membrane receptors that are specific for invading pathogens. There are two distinct subsets of lymphocytes, T and B lymphocytes which differ in function and protein products. The T lymphocytes can be further subdivided into CD4 or T helper lymphocytes and CD8 cells or T-cytotoxic lymphocytes.

Lymphocytes are the cells responsible for the specific immune response to foreign antigens and these cells are responsible for the immunological memory that is necessary for a faster, stronger immune response when exposed to the same antigen at a later time. In the present study, CD4 and CD8 T lymphocyte populations in the peripheral blood of bovines were studied. CD4 cells were present in greater number compared to the CD8 cells in blood and further, the ratio of CD4 to CD8 obtained from blood was consistently more than one (>1) throughout the study. These findings are in agreement with the previous studies on the CD4 to CD8 ratio in the bovine peripheral blood (Oksenberg*etal*., 1985; Yang *etal*., 1988; Park *etal*., 1992 and Shafer and Sordillo, 1996).

Flow cytometric analysis revealed a significant difference ($P<0.01$) in the percentage of CD4 cells between *S.aureus* BF vaccinated and control group on 30^{th} and 60^{th} day and highly significant on 90^{th} and 120^{th} day ($P<0.001$). CD4 cells marginally increased on day 30^{th}, 60^{th}, 90^{th} and significantly increased ($P<0.01$) at day 120^{th} in the *S.aureus* BF vaccinated group compared to *S.aureus* FC vaccinated group. The CD4 cells or T helper cells produce a variety of cytokines which lead to Th1 and Th2 immune responses and antibody production from B cells (Mosmann *et al*., 1986). Moreover, increased number of CD4 may be associated with the initiation of another type of cell mediated immune response (CMI), delayed type hypersensitivity (DTH), which is effective in eliminating intracellular pathogens (Lee *et al*., 2005). The increase of CD4 cells coincided with the elevated IgG level in serum, observed by indirect ELISA indicating the requirement of CD4 cells for IgG production.

Further, the flow cytometric analysis of the present study also revealed the difference in value between *S.aureus* BF vaccinated and control group for CD8 cells produced was significant on 60^{th} day ($P<0.01$) and highly significant ($P<0.001$) on days 90 and on 120 respectively. However, the comparison of CD8 profiles between *S.aureus* BF and FC vaccinated groups showed the significant increase in CD8 cells on day 120 as reported in case of CD4 assay. Further, CD8 T lymphocytes also known as cytotoxic T cells recognize and eliminate altered self-cells via antigen presentation in conjunction with MHC class I molecules. Therefore, cytotoxic T cells may act as scavengers, removing old or damaged secretary cells, the presence of which could increase the susceptibility of mammary gland to infections (Taylor *et al*., 1994). Cytotoxicity, as a general mechanism for pathogen control can involve apoptosis of infected cells through Fas/Fas I interaction or lysis/apoptosis of infected cell resulting from release of cytotoxic granule protein (Flynn, 2006). In the present

study, as per the flow cytometric data, the CD4:CD8 ratio was almost maintained at 2:1 level throughout the study but the percentage of cells increased up to13.4 and 14.4 for CD4 cells and 9.8 and 10.2 for CD8 cells respectively at 90 and 120 days after *S.aureus* BF vaccination and increased up to 11.2 and 10.1 for CD4 cells and 6.4 to 6.4 for CD8 cells respectively at 90^{th} and 120^{th} days after *S.aureus* FC vaccination.

Staphylococcus aureus BF based vaccination showed a significant enhancement in CD4 and CD8 cells. The percentage of CD4 and CD8 cells increased significantly ($P<0.001$) in the *S.aureus* BF vaccinated groups than in the control group and further, in comparison with *S.aureus* FC vaccine, the percentage of CD4 and CD8 cell population found to be marginally increased on 60 and 90 days and significantly ($P<0.01$) increased on 120 days of post vaccination. These findings are in very much conformity with Gomez *et al*. (2002) who also observed the increase of CD4 and CD8 T cells in association of immunization with a live attenuated *S.aureus* mutant to protect the mouse mammary gland from infection. They reported CD4and CD8T cells appear tobe the main lymphocyte subpopulations involved in this response and they suggested that IFN-γ production induced by intramammary immunization may play a pivotal role in the eradication of intracellular staphylococci.In another study by Chandrashekara (2009) who also reported the increased levels of CD4 and CD8 cells in the *E.coli* BF vaccinated cows by flow cytometry.

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