

IMPACT OF DEXTRAN SULPHATE ADJUVANTED *S. aureus* VACCINE AGAINST THE CONTROL OF MASTITIS IN LACTATING DAIRY BUFFALOES IN PAKISTAN

Muhammad Kashif^{1,*}, Amar Nasir¹, Muhammad Rizwan², Asghar Hussain³, Usman Waheed⁴, Aziz-ur-Rehman⁴, Arbab Sikandar⁵ and Hafiz Muhammad Khizer Aziz¹

Received: 15 April 2021

Accepted: 22 December 2023

ABSTRACT

The present study was conducted to evaluate two *S. aureus* vaccines in 100 mastitis free lactating buffaloes, dividing into 2 equal groups (B₁, B₂). The animals of B₁ and B₂ were administered with 2 shots of live attenuated and Dextran sulphate adjuvanted *S. aureus* vaccine at 15 days sequentially. The evaluation was done with different parameters i.e., serum and whey antibody titers, somatic cell count, milk fat %, milk protein, milk yield, vaccine efficacy, cost-benefit analysis, and colony count. There was a peak of geometric mean antibody titer 291 and 58 in LSAV while its climax 363 and 90 in DSAV at 2 and 6 months of study. In whey this level almost remained the same in both groups. In B₁ and B₂, somatic cell count kept on decreasing from day zero to the end of study. There was a non-significant difference in milk yield and fat percentage between the 2 groups.

Milk protein concentration was significantly different between these groups and was better in B₁ than B₂. The surf field mastitis test-based quarter point prevalence decreased at 180 days in LSAV and DSAV. In California mastitis test based, a significant decreased value was shown in both groups. Pre-vaccination and post-vaccination colony count of *S. aureus* was more in LSAV than in DSAV. Moreover, the preventative efficacy and cost benefit ratio of DSAV was more excellent as compared to LSAV.

Keywords: *Bubalus bubalis*, buffaloes, lactating animals, somatic cell count, protein, vaccine

INTRODUCTION

Mastitis is the inflammation of milk producing organs (Biswadeep *et al.*, 2015). It affects

¹Department of Clinical Sciences, Sub Campus-Jhang, University of Veterinary and Animal Sciences, Lahore, Pakistan, *E-mail: muhammad.kashif@uvas.edu.pk

²Department of Clinical Sciences, Bahauddin Zakariya University, Punjab, Pakistan

³Department of Animal Production, Sub Campus-Jhang, University of Veterinary and Animal Sciences, Lahore, Pakistan

⁴Department of Pathobiology, Sub Campus-Jhang, University of Veterinary and Animal Sciences, Lahore, Pakistan

⁵Department of Basic Sciences, Sub Campus-Jhang, University of Veterinary and Animal Sciences, Lahore, Pakistan

all species of animals and becomes dangerous for animal, young ones and owner (Hoque *et al.*, 2015). This disease is extremely important in dairy animals especially in cattle and buffaloes (Ashfaq *et al.*, 2015) and deteriorate the quality (Welderufael *et al.*, 2016) along with reduction in quantity of milk (Huirong *et al.*, 2019) making an unprofitable dairying for the owner (Rohmeier *et al.*, 2020). The intake of sub-standard milk carries diseases to the consumers i.e., tuberculosis, brucellosis, streptococcal sore throat, staphylococcal toxemia, scarlet fever, gastro-enteritis, and leptospirosis (Sharif and Muhammad, 2009). It is also injurious to the health of calves and retards their growth (Radostits *et al.*, 2007). A study conducted in India indicated, the loss of 52.9 crores Rupees because of blind teats (Amer *et al.*, 2018). Mastitis causes 53 billion US dollar per annum all over the world (Christian *et al.*, 2020), 2 billion US dollar in USA and 300 million pounds in the UK (Piepers *et al.*, 2016). This disease assumes two forms viz. clinical form and sub-clinical form (Radostits *et al.*, 2007), later is 15 to 40 times is more common than clinical form (Vakkamaki *et al.*, 2017). In Pakistan, buffalo is a premier dairy animal and produces more than 75% milk to be used all over the country (Sarwar *et al.*, 2002). Buffalo is affected with mastitis which is caused by many bacteria (Hogeveen *et al.*, 2011) but *S. aureus* is the most common organism isolated (Bachaya *et al.*, 2011) and mostly causes sub-clinical mastitis leading to clinical failure up sometimes (Yousaf *et al.*, 2009). This organism foils the effect of antibiotic because of having the multifarious characteristics of resistance development and evading the host's defense (El-jakee *et al.*, 2019). That is why, once entrance into udder, and makes it a permanent resident for ever eating up the parenchyma like termite (Suleiman *et al.*, 2018). The majority of

control measures like hand wash, post-milking teat dipping, prompt treatment, dry therapy and culling are being used to treat mastitis but still disease is widely prevalent. It is dire need to control this disease through the vaccination of animals as other diseases like Hemorrhagic Septicemia and Foot and Mouth disease are being controlled. Dextran adjuvanted *S. aureus* vaccine had proved its worth in our preliminary studies in Rabbits (Shakoor *et al.*, 2006). Therefore, the present study was designed to evaluate two *S. aureus* vaccines (live attenuated and Dextran Sulphate adjuvanted) in lactating buffaloes.

MATERIALS AND METHODS

Isolation and Bio characterization of field isolates

Foremilk samples were collected aseptically from 100 clinically mastitic quarters of buffaloes. These samples were streaked onto blood agar plates containing 5% sheep erythrocytes and staph 110 medium. Colony morphology and haemolytic patterns of isolates were noted. Gram positive, catalase positive, coagulase positive (at 4 h) isolates were identified as *Staphylococcus aureus* (National Mastitis Council, Inc., 1990). A typical alpha, beta hemolytic *S. aureus* field isolate was selected and biotype using a commercially available kit (API -staph identification codebook; 1986: API Analytic products, Division of Sherwood Medical 200 Express Street Plain view New York-11803).

Optimization of cultural conditions

After checking the pathogenicity of a selected vaccinal isolate of *S. aureus*, optimum cultural condition was standardized to get the encapsulated

S. aureus. For this purpose, the selected isolate was grown on blood agar plate Nutrient Broth (NB), modified nutrient broth, brain heart infusion broth (BHIB) and modified BHIB. Modification of NB and BHIB was done by incorporating bubaline sterile whey 10% v/v. Extent of the expression of the pseudocapsule in different cultural conditions was confirmed by autoagglutination method (Bebora *et al.*, 2017).

Determination of expression of Pseudocapsule

Culture of *S. aureus* was centrifuged (1200 x; 10 minutes, 4°C) and the cells were washed once with sterile phosphate buffer saline, pH 7.2, then resuspended in sterile PBS and adjusted spectrophotometrically to a concentration of 5×10^9 cells ml⁻¹. A 0.1 ml aliquot of the suspension was added to each well of a U-boittomed microtiter tray. Then 0.1 ml of sodium hydroxide solution prepared at the various molarities ranging between 0.001M and 0.1M was added to each well. These solutions were made by diluting 1.0M sodium hydroxide with sterile PBS. A PBS control (without sodium hydroxide) was included; the tray was covered, allowed to stand for 16 h. The autoagglutination titer was recorded at the ambient temperature (Abebe *et al.*, 2016).

Preparation of sterile milk whey

One liter milk whey was prepared by rennin precipitation from 1.5 liter milk while collected commercially. A 0.5 mg of rennin was dissolved in 27 ml of saline and the suspension was added to 1.5 liter of delipidized milk. After 30 minutes at 37°C, samples were centrifuged at 10,000 X g for 20 minutes. The whey samples were then sterilized by filtration gradually from 0.45 and 0.22 micrometer membrane filter. Filtered milk whey was stored at 20°C till required.

Preparation of dextrin sulphate adjuvanted vaccine

The selected field isolate of *S. aureus* was grown independently in modified nutrient broth at 37°C for 48 h on an orbital shaker set at 60 rpm under aerobic conditions. After comparing the purity and pseudocapsule elaboration of the culture. The suspension was inactivated with formalin (0.4% v/v), centrifuged (at 6000Xg for 1 h at 4°C), and resuspended in PBS at pH 7.2. Bacterial concentration was adjusted to 10¹⁰ cells/ml spec photometrically. Sodium aside thimerosal and formalin were added as preservatives at final concentrations of 0.001% (w/v), 0.001% (w/v) and 0.4% (v/v), respectively. Incorporation of crude toxin extract of *S. aureus* (Dorota-Anglart *et al.*, 2020). Supernatant fluid was collected from 48 h broth culture of *S. aureus*. The supernatant was separately autoclaved at 121°C for 20 minutes. This preparation was then centrifuged at 6000Xg for 30 minuets 4°C and the supernatant was added to the vaccine preparation at a concentration ration of approximately 5 mg of dry weight per dose. Finally, dextran sulphate (DXS, MW. 500,000; Sigma-Aldrich Co, St. Louis, USA) was added as an adjuvant in the vaccine at a concentration of 50 mg per ml dose (Zeryehun *et al.*, 2017).

Selection of lactating buffaloes

A total 100 lactating *Nilli-Ravi* buffaloes were selected at Livestock Production Research Institute (LPRI) Bahader Nager Okara and divided them into 2 equal groups i.e., B₁ and B₂. The B₁ buffaloes were administered DSAV twice at month interval 5 ml intramuscularly. Serum samples were taken on day zero followed by a monthly intervals till 6 months (180 D). Group B₂ was injected (Placebo, Normal saline) in a similar way along with blood collection monthly till day 180.

Parameters for the evaluation of vaccines

Serum and milk whey antibody titers were measured using standard modified indirect Haemagglutination test (Rehman *et al.*, 2004), Modified somatic cell count (Zeryehun *et al.*, 2017), butter fat concentration (Ismail, 2017), Milk protein concentration, Effect on the milk quantity, Prevalence, Commutative incidence, Serenity of clinical episodes, No of colonies of *S. aureus*, Pre-vaccination (days 0) colony members in the respective groups were compared with those recorded at day 180 post-vaccination to determine the effect of vaccination on colony farming units of *S. aureus*.

Vaccine efficacy

Preventative efficacy of DSAV was calculated at day 120 and 180 post-vaccination as per method described by (Landin *et al.*, 2015). To this end an estimate of the vaccine efficacy was obtained from the odds ratio (OR) using the following formula for estimated an attributable fraction;

$$\text{Estimated Attributable fraction} = \text{OR}-1/\text{OR}.$$

Odds ratio at days 120 and day 180 was calculated by the following formula Odds Ratio (DR) = ad-bc.

Cost-benefit analysis of mastitis control through vaccination

The mean milk yield of each group of buffaloes at each sampling time point starting from booster vaccination was measured to calculate the total milk production over one month period. The monthly milk yield of each group was aggregated to obtain total milk produced in a period between booster vaccination and final sampling time point

(day 180). The difference of milk yield between DSAV and unvaccinated Control group (B_2) was calculated by subtracting the yield from the B_2 from the yield of B_1 . Then the difference was multiplied by the prevailing price of milk per liter at the farm gate to calculate the economic benefit of using each vaccine.

Data analysis

The data thus generated was subjected to statistical analysis using the SAS- 2000 computer program.

RESULTS AND DISCUSSIONS

Isolation and bio characterization of isolates from Mastitis cases

Sixty four out of 100 mastitic samples were found Gram-positive, catalase-positive, coagulase-positive, and alpha Beta hemolysis producing cocci were regarded as the virulent isolates of *S. aureus* as per method described by National Mastitis council, Inc. USA (1990). Out of 64, 30 were found alpha-hemolytic, 30 Beta-hemolytic and 4 alpha Beta hemolytic. A typical alpha Beta *S. aureus* isolate was selected as a candidate for vaccines. The isolate gave 7-digit API-Staph biochemical profile i.e., 6336153; Good identification (97.5%) (API-Staph identification codebook; 1986; API Analytas product, Division of Sherwood Medical 200 Express Street Plainview, New York 11803).

Modified nutrient broth proved as the best pseudocapsule developing medium in which autoagglutination was observed in molar concentration solution of NaOH from 0.04 to 0.001 while partial agglutination was found in modified brain heart infusion broth in molar concentration solution of NaOH between 0.04 and 0.03 Dextran

Sulphate adjuvanted *S. aureus* vaccine (DAV), elicited higher serum antibody titers than the control (B₂). These monthly values were 4,235, 363, 302, 258, 193 and 90 starting from day 0 to day 180. For Group B₂; IHA indirect Hemagglutination antibody titers (GMT) were 5.3 at day 0, which remained almost same till the termination of trail.

Serum antibody titers

DAV elicited higher serum antibody titers than the Control (B₂). These monthly values were 1, 135, 363, 302, 258, 198 and 90s starting from day 0 to day 180. For Group B₂, IHA antibody titers (GMT) was 3.5 at day 0, which remained almost same till the termination of trial. These findings are in line with those of (Nyman *et al.*, 2018). A higher IHA antibody titer shown by DAV may be attributed to the addition of an adjuvant (DXS) which has a specific ability for triggering the IgG₂ response as mentioned by various workers (Kerro Dego *et al.*, 2020) who concluded that Dextran Sulphate is a useful adjuvant with *S. aureus* vaccine in both quantity and quality of antibody, but it was recognized that antibody response was not adequately durable. IgG₂ is cytophilic for neutrophil and confers enhanced phagocytic capacity to the cells. Milk whey IHA antibody titers were 3.5, 4, 3.2, 2.6, 2.3, and 2.0, respectively on every monthly interval in DAV group. While it was 3.2 at day 0 and remained almost at the same level during the period of 180 days. There was non-significant difference between the two groups. The whey antibody titers in B₁ Group remained between (2 to 4 GMT) because of being no infection during study period and was almost the same in B₂ (UC).

The mean somatic count was 11.06±4.01 million on day 0 and continued to wane on successive monthly intervals i.e., 5.11±3.64, 2.54±1.53, 2.42±2.04, 2.18±1.42, 2.26±1.59

respectively and was 4.11±1.79 at the end of trail (180 day). In the unvaccinated Control group, it was 13.52±4.15 x 10⁵ /ml at day zero and declined till day 60 followed by a slight rise to 10.26±3.70 at day 90. It decreased to 7.15±1.92 x 10⁵/ ml at day 150 and rose moderately to 9.00±3.02 at day 180. The difference was highly significant (P<0.01) between the two groups. SCC is a standard tool for monitoring mastitis and increase in sub-clinical mastitis caused by *S. aureus*. In B₁, low SCC may be ascribed to the absence of infection during the study period and B₂ group had high SCC due to intermittent infection. These findings are in accordance with the results of (Enger *et al.*, 2018; Abdi *et al.*, 2018).

In Group B₁, the mean fat percentage was 6.01±0.85 at day 0 and corresponding values were 6.22±0.80, 6.11±0.57, 6.03±0.55, 6.03±0.57, 6.25±0.55 and 6.70±0.48, respectively on 30, 60, 90, 120, 150 and 180 days. In Group B₂ (unvaccinated control) it was 6.00±0.38 at day zero and kept on decreasing to 5.0±0.19 at day 150 and was 5.5±0.45 at day 180. There was a significant difference between the two groups. Milk fat was low just in the beginning in the bottom-line data and then began to increase noted at monthly intervals. This is not in agreement with the findings of the workers (Biswadeep *et al.*, 2015) who found no observable effect on fat in milk.

In animals vaccinated with Dextran Sulphate adjuvanted vaccine (B₁) milk protein concentration was 3.34±0.32 at day zero, which remained unchanged till day 30 and rose slightly to 3.36±0.21 at day 60 followed by a slight dip at day 90 and 120. A non-significant increase (P<0.05) was recorded at day 150 and 180 post- vaccination. In Group B₂ (Control) a constant decline in milk protein concentration was recorded from day 0 to day 120. A non-significant increase was registered

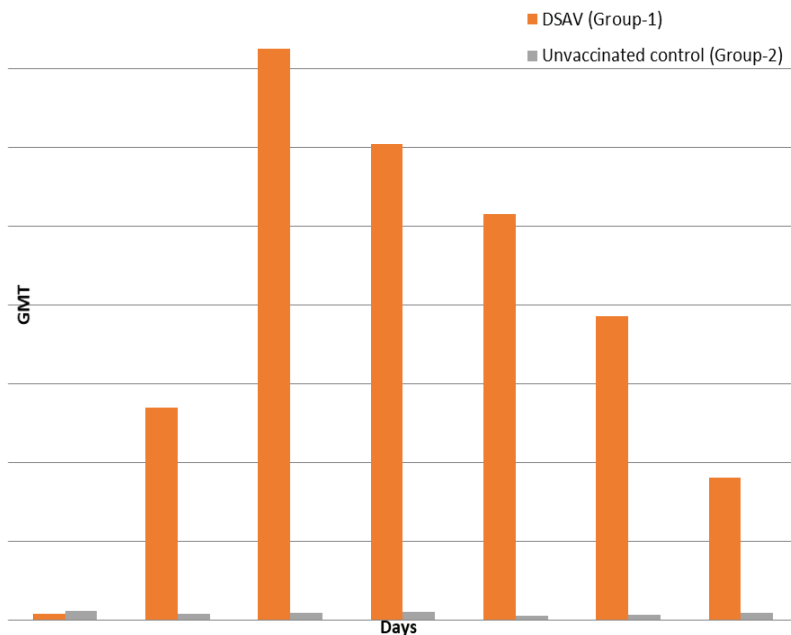


Figure 1. Comparative geometric mean serum Iha antibody titers in lactating buffaloes vaccinated twice during postpartum period with dextran sulphate adjuvanted *S. Aureus* vaccine (DSAV) and unvaccinated Control group.

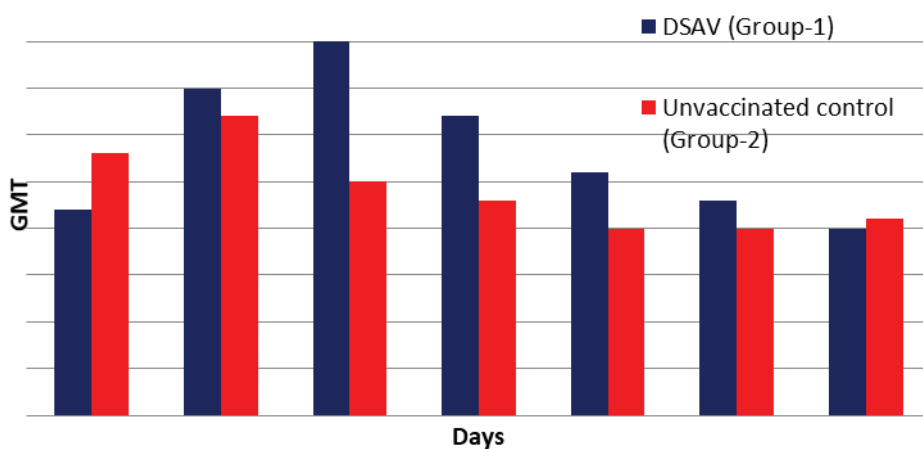


Figure 2. Geometric mean milk whey IHA antibody titers in lactating buffaloes vaccinated twice during postpartum period with dextran sulphate adjuvanted *S. aureus* vaccine (DSAV) and unvaccinated Control group.

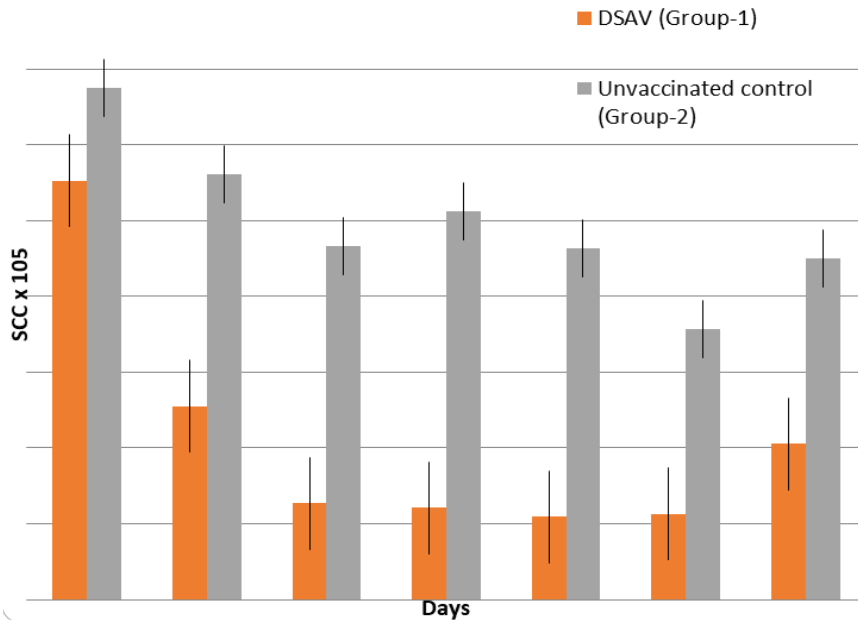


Figure 3. Comparative milk somatic cell count (Mean \pm SD; 10⁵/ml of milk) in lactating buffaloes vaccinated twice during postpartum period with dextran sulphate adjuvanted *S. aureus* vaccine and unvaccinated Control group.

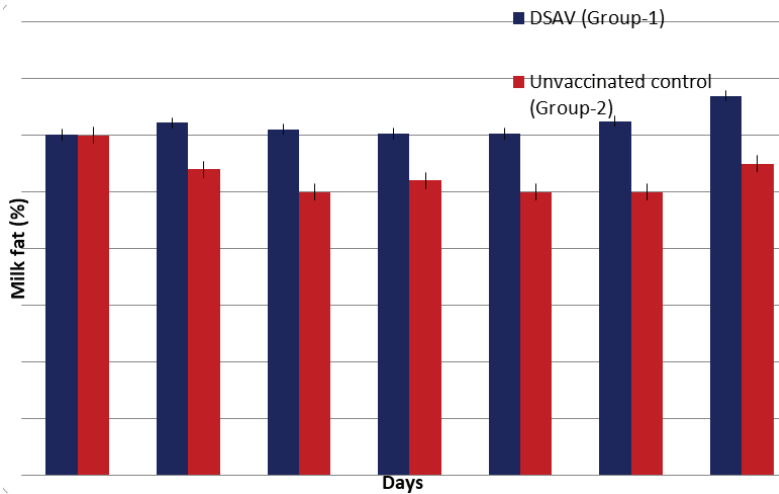


Figure 4. Comparative milk fat percentage (Mean \pm SD) in lactating buffaloes vaccinated twice during postpartum period with dextran sulphate adjuvanted *S. aureus* vaccine and unvaccinated Control group.

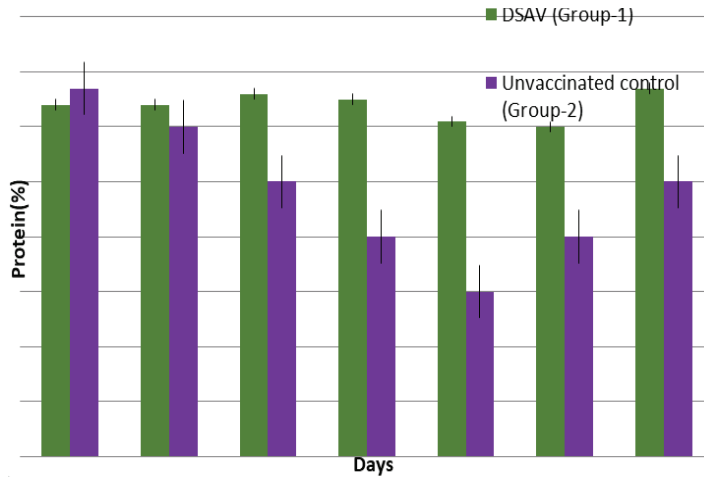


Figure 5. Milk protein percentage (Mean \pm SD) in lactating buffaloes vaccinated twice during postpartum period with dextran sulphate adjuvanted *S. aureus* vaccine and unvaccinated Control group.

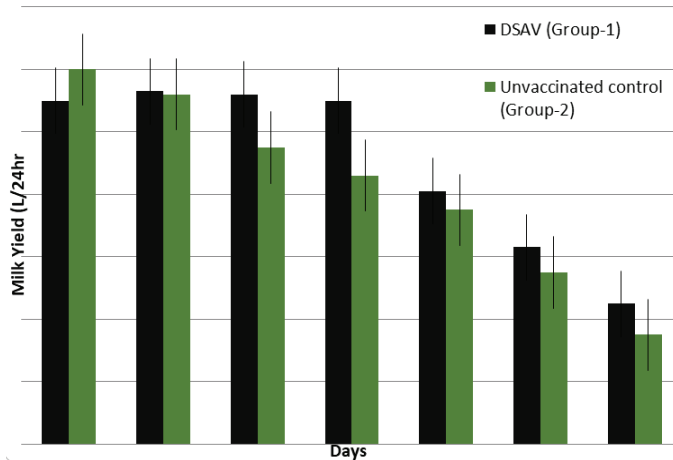


Figure 6. Milk protein percentage (Mean \pm SD) in lactating buffaloes vaccinated twice during postpartum period with dextran sulphate adjuvanted *S. aureus* vaccine and unvaccinated Control group.

at day 150 and 180.

In animals vaccinated with Dextran Sulphate adjuvanted vaccine (B_1) mean milk yield stood at 11.0 ± 1.85 at the start of the trail (day 0). Milk yield peaked at day 30 post vaccination followed by a declining trend towards the end of the study. In the unvaccinated buffaloes (Placebo, B_2) the mean milk yield in the beginning was 12.0 ± 0.39 which started declining towards the termination of the study. Difference between the two groups was significant ($P < 0.05$). These significant results are inline with the findings with the (Hogeveen *et al.*, 2011; Motaung *et al.*, 2017; Amer *et al.*, 2018).

Comparison between vaccinated (B_1) and unvaccinated Control (B_2) group in terms of reactivity in California mastitis test (CMT) indicated that DSAV affected an appreciable reduction in quarter point prevalence till day 120. The percentage reduction in quarter point prevalence during this period ranged from 13 to 15. In Group (B_2) prevalence of CMT positive quarters remained almost unchanged throughout the course of trail. Microbiological examination of quarter foremilk samples revealed that at the initiation of trail (day 0) 29.2 and 28.5% quarter harbored *S. aureus*. Comparison indication an appreciable reduction in infection rate till sampling day 120 and was 14 to 17. While in group B_2 waxes and wanes in prevalence of *S. aureus* infection were recorded throughout the period. It is inline with the findings of (Bebora *et al.*, 2017; El-jakee *et al.*, 2019), who recorded an appreciable reduction in colony counts for *S. aureus* in milk of vaccinated buffaloes. During first 4 months (day 0 to 120) post vaccination, cumulative incidence of *S. aureus* intramammary infection was 0.063 while it was 1.74 folds higher than B_1 . During the last two months of study (day 121 to 180) in B_1 and B_2 , a relatively more frequent occurrence of new cases

of *S. aureus* intramammary infection was recorded and were 0.14 and 0.139 in B_1 and B_2 respectively. Quarter point prevalence (%) of *S. aureus* intramammary infection based on microbiological examination of milk was low in B_1 as compared to B_2 (UC). It is inline with the findings of many workers (Zeryehun *et al.*, 2017; Ismail, 2017; Suleiman *et al.*, 2018), who concluded while using Dextran Sulphate in cows and sheep that incidence and prevalence of *S. aureus* mastitis was reduced significantly.

The mean clinical severity score at the outset decreased from 2 to 1.68 at the termination of trail in buffaloes vaccinated with DSAV while it increased from 1.75 to 1.84 at the end in unvaccinated control. It is consistent with the findings of (Vakkamäki *et al.*, 2017; Tashakkori *et al.*, 2020; Hernández-Castellano, *et al.*, 2017; Kusebauch *et al.*, 2018; Landin *et al.*, 2015; Freick *et al.*, 2016; Bradley *et al.*, 2015; Butt, 2006; Athar, 2007), who concluded a decline in the severity of *S. aureus* mastitis while using DSAV in sheep, cows, and buffaloes.

So, it was concluded that Dextran Sulphate Adjuvanted Vaccine (DSAV) affected a significant reduction in *S. aureus* colony count over 180-day period of trial. Contrarily, statistically non-significant differences were recorded in Control group (B_2). Pre-vaccination and post-vaccination colony count of *S. aureus* was more in LSAV than in DSAV. Moreover, the preventative efficacy and cost benefit ratio of DSAV was more excellent as compared to LSAV.

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