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RESEARCH ARTICLE

Evaluating the Effectiveness of Multidrug Resistant *Staphylococcus aureus* Mastitis Vaccines in Dairy Cattle

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A B S T R A C T

Indiscriminate use of antibiotics to treat *Staphylococcus aureus* bovine mastitis may impair mammary glands immune function and develop bacterial resistance. Consequently, developing alternative remedies to treat mastitis is imperative. Current study evaluated plain multidrug resistant *S. aureus* vaccine (PMRSAV), Montanide oil adjuvanted multidrug resistant *S. aureus* vaccine (MMRSAV), and Aluminum hydroxide adjuvanted multidrug resistant *S. aureus* vaccine (AMRSAV) in lactating dairy cattle. Serum samples through IHA revealed that MMRSAV provoked maximum antibody titer at day 90 (274.4±0.41) whereas AMRSAV (147±0.46) and PMRSAV (78.8±0.44) produced highest antibody response at day 60. The cumulative mean serum IHA antibody titer was recorded highest for MMRSAV (148.4) than AMRSAV (78.4) and PMSAV (34.1). The peak milk whey antibody titer was seen at day 60 for PMRSAV (9±0.23) and AMRSAV (14.7±0.24) whereas it was observed highest at day 90 in MMRSAV (26.2±0.25). Somatic cell count (SSC: $\times 10^5$ mL^{-1}) observed decline trend in all the vaccinated cattle until day 90 for PMRSAV (1.449±0.219), day 120 for MMRSAV (1.201±0.097) and AMRSAV (1.327±0.104). During study period, the highest quarter-based incidence was observed in control group (25%) followed by PMRSAV (17.5%), AMRSAV (10%) and MMRSAV (5%) resulting incidence reduction (80%) for MMRSAV followed by AMRSAV (60%) and PMRSAV (30%). Post challenge lowest overall mean somatic cell count was recorded in group vaccinated with MMRSAV (2.323±0.46) followed by AMRSAV (3.006±0.43), PMRSAV (3.759±0.82) and unvaccinated control group D (7.798±1.11). The study concluded that MMRSAV is the most effective in preventing multidrug resistant *S. aureus* mastitis in dairy cattle.

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INTRODUCTION

Staphylococcus aureus, the most common causative agent of mastitis in dairy cows (Merrill *et al.,* 2019) continues to be a major challenge for dairy industry economics by affecting animal productivity (Heikkilä *et al.,* 2018). Being the natural part of skin flora, *S. aureus* is difficult to eradicate from the herd. Moreover, endotoxin and biofilm contribute to its pathogenicity in addition to adherence to mammary epithelial tissue. Additionally, it produces various enzymes such as proteases, lipases and elastases during infection and destroy leukocyte due to cell membrane perforation by leukocidin (Algharib *et al.,* 2020). The efficacy of treatment against this organism is often disappointing, as it causes extensive damage to the glandular tissues of the udder and therefore most antimicrobials cannot penetrate all infected sites (Pu *et al.,* 2014). Cure rates for treating *S. aureus* mastitis with intramammary infusion or parenteral antimicrobial administration are markedly less than satisfactory, and infections commonly persist throughout the life of the cow.

Multi-drug resistance (MDR) has become a growing problem in the treatment of infectious diseases and the widespread use of broad-spectrum antibiotics has resulted in the development of antibiotic resistance by numerous human and animal bacterial pathogens. As a result, microorganisms are resistant to multiple antibiotics causing ongoing economic losses in dairy production. Multidrug resistance has emerged as a result of the indiscriminate use of antibiotics to treat *S. aureus* bovine

mastitis causing huge economic losses in dairy production (Yuan *et al.,* 2017). Hence it is critical to develop alternative therapies and control strategies for mastitis (Peralta *et al.,* 2020). Researchers are always attempting to develop effective mastitis control strategies and vaccinating cows during lactation may be effective in boosting their resistance to mammary gland infection (Mohyuddin *et al.*, 2020). The use of vaccines in farm animals significantly reduces antibiotic consumption and reduces the risk of developing AMR (Hoelzer *et al.,* 2018). In addition, decreased transmission of pathogens and infections in vaccinated entities significantly reduce antibiotic therapies and decrease the circulation of resistant strains (Lipsitch and Siber, 2016).

Vaccination against *S. aureus* mastitis has been investigated for many years; however, no vaccine has consistently prevented *S. aureus* infections. Vaccines against *S. aureus* have variable outcomes depending upon immunization, route and adjuvant used. Moreover, vaccines against *S. aureus* mastitis with optimal efficacy and reliability are still being investigated.

Controlling mastitis in Pakistan gets more challenging when there is no mastitis control program in place. Due to small herd size, farmer illiteracy, and a lack of milk quality premium, standard mastitis management techniques are difficult to implement. Due to widespread antibiotic resistance, vaccination against the most prevalent mastitis pathogen is the suitable option to curtail mastitis losses (Yousaf *et al.,* 2009). In Pakistan, many researchers (Butt 2006; Shakoor 2006; Athar, 2007; Ahmad and Muhammad, 2008) have reported about variable protection by vaccine against mastitis but not known about multiple drug resistance *S. aureus* (MDRSA) mastitis vaccine evaluation in cattle. Moreover, vaccines against *S. aureus* mastitis with optimal efficacy and reliability are still being investigated. The development of an effective vaccine to prevent *S. aureus* mastitis would enhance animal welfare, minimize antibiotic use and improve the economics and efficiency of milk production (Alabdullah *et al.,* 2021). Hence, the current study evaluated the immunogenic response of Plain, Montanide oil adjuvanted and Aluminum hydroxide adjuvanted MDR *S. aureus* vaccines in lactating dairy cattle.

MATERIALS AND METHODS

Source of MDR *S. aureus* isolate: Purified and molecularly characterized isolate of MDR *S. aureus* of bovine origin was procured from funded research project "Development of polyvalent vaccines for the control of mastitis in dairy cattle" at Animal Health Research Laboratory of Department of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore-Pakistan {Grant No. 638 funded by Punjab Agricultural Research Board (PARB)}.

Preparation of plain and adjuvanted MDRSA mastitis vaccines: Plain and adjuvanted vaccines were prepared using field isolate of multidrug resistant *S. aureus* (MDRSA) of bovine origin. The vaccines were prepared according to the procedures and protocol as described in previous studies (Ahmad and Muhammad, 2008; Giraudo *et al.,*1997). Briefly, fresh blood agar culture of MDRSA was grown in modified nutrient broth (modified by adding 10% w/v bovine whey) for enhanced encapsulation of MDRSA at 37ºC for 48h on an orbital shaker set at 60 rpm under aerobic conditions. Confirming the purity and presence of pseudocapsule, MDRSA suspension was inactivated with formalin (0.4% v/v) for 24 hrs and centrifuged at 6000xg for 1 hour at 4ºC. The supernatant was collected, autoclaved and stored at 4ºC for further use as toxoid. MDRSA pellet was re-suspended in PBS and concentration was adjusted to $1x10^{10}$ cells/mL (the concentration that provoked highest immune response in rabbits) spectrophotometrically (Hirsch and Strauss 1964). Formalin (0.4% w/v), sodium azide (0.004% w/v) and thimerosal sodium (0.004% w/v) were added as preservatives. Crude toxin extract was also added at 5mg of dry matter per dose. This suspension was used as plain MDRSA vaccine. For Montanide adjuvanted vaccines, equal volume of Montanide oil (Seppic France) was mixed in plain MDRSA vaccine and homogenized using Ultratourex. Likewise, aluminum hydroxide gel was prepared and added at 3.5% to plain MDRSA vaccine to get aluminum hydroxide adjuvanted MDRSA vaccine. Phosphate buffer saline (pH 7.2) supplemented with formalin (0.4%) sodium azide (0.004%) and thimerosal sodium (0.004%) used as placebo. These vaccines were found sterile, safe, and provoked strong humoral immune repose in laboratory animals.

Evaluation of MDRSA mastitis vaccines in lactating dairy cattle: MDR *Staphylococcus aureus* mastitis vaccines were injected in lactating dairy cattle reared at Training and Research Demonstration Farm Program (TRDFP) of the University of Veterinary and Animal Science, Ravi Campus Pattoki. These forty mastitis free cattle in their first two months of lactation were randomly divided into four groups (A-D) having 10 cattle in each group. Group A was injected with plain vaccine (PMRSAV) 5ml SC, group B with Montanide oil adjuvanted vaccine (MMRSAV) 5ml I/M, cattle of group C were given Aluminum hydroxide adjuvanted vaccine (AMRSAV) 5ml SC whereas group D served as control being administered Placebo 5ml SC. At day 21 of priming, a booster dose was given to cattle in each group. Serum samples were collected from each group after every month for up to 6 month and serum antibody titers were measured against MDRSA using IHA test as described by (Rahman *et al.,* 2005). In addition to this, monthly milk whey samples were also collected and evaluated for antibody titers using the IHA test (Rahman *et al.,* 2005).

Quarter foremilk samples from the vaccinated and control groups were taken monthly for 6 months and somatic cell quantified using an electronic somatic cell counter (Lactoscan, Milkotronic, Bulgaria).

Milk data was collected from all groups on monthly basis in order to compute and compare the mean milk yield of the vaccinated and control groups. The LSCCS approach (Kirk 1984; Shakoor *et al.,* 2006; Athar 2007) was also used to calculate anticipated milk loss monthly over six months using somatic cell counts.

Quarter milk samples were taken from each group's cattle to determine the incidence of mastitis using the California Mastitis and the surf field mastitis tests

(Muhammad *et al.,* 1995) at days 0, 30, 60, 90, 120, 150 and 180.

Challenge protection was evaluated two week after booster vaccination on the basis of somatic cell count at alternate days for 14 days post challenge after immersion the teats of five animals from each group of cattle in a live inoculum of MDRSA in peptone solution (0.5% w/v peptone and 0.5% w/w NaCl) containing 10^{10}cfu ml-1 (Boddie and Nickerson 2002) soon after milking. Moreover, Dinitrochlorobenzene (DNCB) test was also performed on 6 animals (two from each group) to assess cell-mediated immune response. Two 3 cm diameter areas were marked with the help of a metal ring in the neck region 15 cm anterior to the shoulder blade. The areas were clipped out with the help of scissors. The metal ring was then placed over the cut-out area and DNCB was slowly dropped as a 2% solution in acetone, i.e. 2-3 drops at a time using a 1 ml insulin syringe fitted with a hypodermic needle. The solution was immediately blown dry to prevent it from running off to the next region. The minimum dose and the number of applications was 0.5 ml of 2% DNCB in acetone for 3 consecutive days. All sensitized animals were challenged at two sites on day 14 on the opposite side. The challenged dose was 0.5 ml of 2% DNCB in acetone. Response was judged by measuring skin thickness before and 24 and 48 hours after exposure. The same experimental procedure repeated with 0.5 ml of acetone without DNCB served as a control (Brummerstedt and Basse 1973; Shakoor 2006; Athar 2007).

Statistical analysis: Geometric mean titers were calculated and subjected to statistical analysis by ANOVA using SPSS version 21.00. The level of significance between the groups was determined using the Dunckun Mutiple Ranges test. A probability level $P < 0.05$ was considered statistically significant.

RESULTS

Geometric mean IHA antibody titers (GMT) taken at monthly intervals from serum of vaccinated and control groups of lactating cattle are presented in (Fig. 1). All the vaccines viz. PMRSAV, MMRSAV and AMRSAV resulted in GMT values of 27.9±0.38, 42.2±0.33 and 39.4±0.37, respectively at day 30 with further increment after booster resulting in GMT at day 60 as 78.8±0.44, 194±0.39 and 147±0.46. The group B had a maximum geometric mean antibody titer of 274.4±0.41 at day 90 whereas group A and C yielded highest antibody concentration of 78.8±0.44 and 147±0.46 at day 60 respectively. During the span of day 90 to day 180, GMT against the vaccines showed downward trend although the titers at day 180 were 10.5±0.4, 147±0.27 and 55.7±0.2 for groups A, B and C, respectively concluding significantly increase ($P<0.05$) in GMT from for all three vaccine groups. Contrary to this, serum antibody titers in unvaccinated control group D did not vary significantly from the baseline titer throughout the study period.

The cumulative mean serum IHA antibody titer of group B (MMRSAV) was higher (148.4) than other groups. The cattle in group C (AMRSAV) resulted in GMT (78.4) while group A (PMRSAV) turned out GMT as 34.1 (Fig. 2).

Milk whey antibody titers in vaccinated and control groups of cattle against MDRSA are shown in (Fig. 3). IHA results revealed that at day 30 post primary inoculations of vaccines (PMRSAV, MMRSAV, and AMRSAV) resulted in milk whey GMT values of 5.5±0.22, 8±0.22 and 6.2±0.24, respectively. The peak milk whey antibody titer was observed in cattle of groups A and C at day 60 (9±0.23 and 14.7±0.24, respectively), whereas in group B it was 26.2±0.25 at day 90. At day 180, milk whey GMT was 6.8±0.2, 16.1±0.17, and 10±0.2 in cattle of group A, B and C, respectively. Hence, a significant ($P<0.05$) increase in milk whey GMT was observed in vaccinated groups. On the other hand, milk whey antibody titers in unvaccinated control groups remained the almost the same throughout the course of experiment as recorded at start of the trial.

Cumulative mean milk whey antibody titers were highest in group B (15.7) followed by the group C and A (9.5 and 6.5 respectively) (Fig. 4).

Somatic cell count (SSC: $\times 10^5$ mL^{-1}) ranged between 1.624±0.223 to 1.794±0.114 for all the cattle groups at start of the study but thereafter decline trend in the somatic cell count was observed in all the vaccinated cattle until day 90 for group A (1.449±0.219), day 120 for group B (1.201±0.097) and group C (1.327±0.104) (Fig. 5). In unvaccinated control group D, no decline trend was observed rather it continued rising from day 0 (1.624±0.223) till end (2.941±0.386) and highly significant difference ($P<0.05$) was observed between control and vaccinated groups at the end of study. The mean somatic cell count during the span of six months was significantly lower ($P<0.05$) among vaccinated groups A, B and C (1.567±0.220, 1.391±0.100 and 1.451±0.116 respectively) as compared to the control group D (2.254±0.306) (Fig. 6). When compared based on reduction in mean SSC from those in control group, maximum reduction was observed in cattle of group B (38.29%) followed by the group C (35.63%), whereas cattle of group A resulted in least reduction (30.48%) which was significant ($P<0.05$) than the other vaccinated groups.

The mean milk yield (liters per 24hrs) in vaccinated and control group of cattle showed a decreasing trend in all the groups (Fig. 7). When compared on the basis of overall milk production during six months, the highest mean milk yield was recorded in the group B of the cattle (23.00±1.1) followed by the group C (22.47±1.1) and group A (21.91±1.1) which differ significantly ($P<0.05$) among them. During six-month trial period, the unvaccinated cattle group D resulted in significantly less milk yield than those of the group A (PMRSAV), B (MMRSAV) and C (AMRSAV) during the span of 6 months' study period (Fig. 8). Furthermore, while comparing milk production between all of the groups; it was observed that cattle groups vaccinated with MMRSAV, AMRSAV, and PMRSAV produced more milk (333, 237.6 and 136.8 respectively) (Fig. 9) during the trial period than the unvaccinated control group D.

Estimated milk loss using Linear somatic cell count score method (Kirk 1984; Shakoor *et al.* 2006a; Athar 2007) presented in Fig. 10 revealed a total milk loss in cattle of group B and C as 204 and 224.4 Liters, respectively. On the other hand, milk loss in cattle of

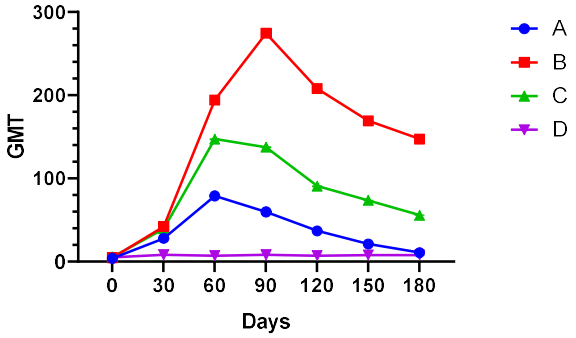

Fig. 1: Geometric mean antibody titers against MDRSA vaccines in cattle.

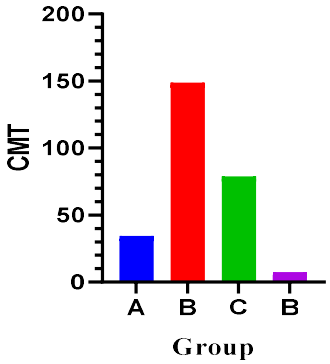

Fig. 2: Cumulative mean antibody titer of vaccine groups.

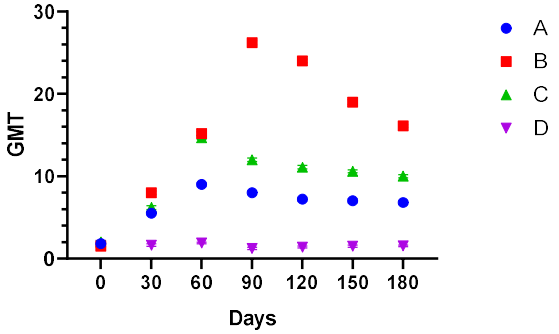

Fig. 3: Milk whey geometric mean titer against MDRSA vaccines.

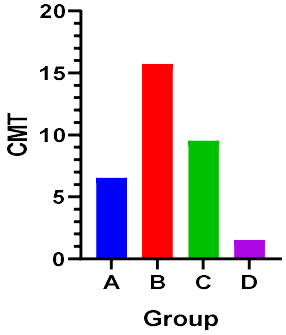

Fig. 4: Milk whey cumulative mean antibody titer of vaccines.

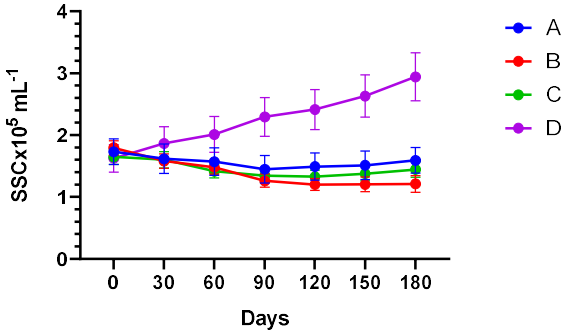

Fig. 5: Somatic cell count of cattle groups against MDRSA vaccines.

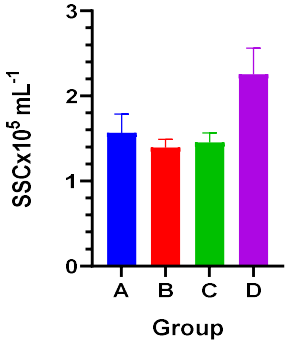

Fig. 6: Mean somatic cell count of cattle groups.

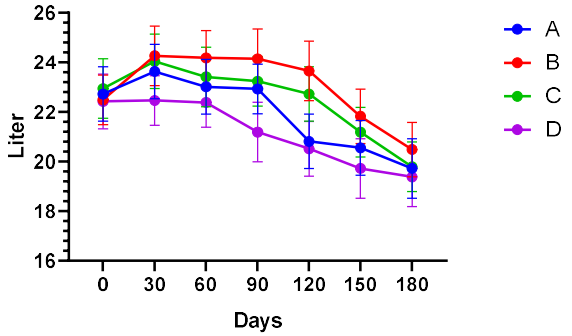

Fig. 7: Milk yield of cattle groups during six months

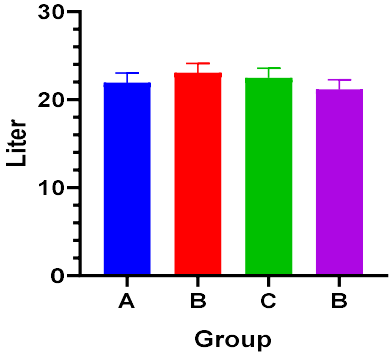

Fig. 8: Mean milk yield of cattle group during six months.

Table 1: Quarter based incidence (%) in vaccinated and control cattle groups at different days post vaccination

Group	Vaccine	Quarter based incidence (%) of mastitis at days post vaccination in cattle							Cumulative incidence (%)
		Day 0	Day 30	Day 60	Day 90	Day 120	Day 150	Day 180	
A	PMRSAV	0	2.5	2.5	2.5	2.5	2.5	5	17.5
B	MMRSAV	0	2.5	0	0	0	0	2.5	5
C	AMRSAV	0	0	2.5	0	2.5	2.5	2.5	10
D	UC	0	2.5	2.5	5	5	5	5	25

PMRSAV= *Plain multidrug resistant S. aureus vaccine*, MMRSAV= *Montanide adjuvanted multidrug resistant S. aureus vaccine*, AMRSAV= *Aluminum hydroxide adjuvanted multidrug resistant S. aureus vaccine.*

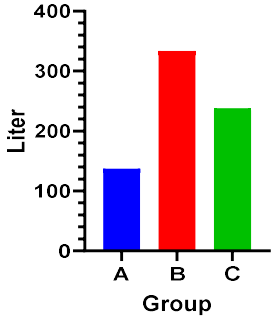

Fig. 9: More milk produced than control group.

groups A and D was 285.6 L 306 L, respectively. Statistical analysis revealed that milk losses in cattle of unvaccinated control groups D were significantly higher ($P<0.05$) compared to vaccinated groups A, B, and C. Likewise, when compared vaccinated groups, the milk losses of cattle in group A were significantly higher ($P<0.05$) than group B and C.

During 6 months of the study period, CMT was routinely conducted at monthly interval for detection of new quarters showing positive reaction (Table 2). The percent incidence rate of mastitis calculated on the basis of 6 months showed that there was significantly higher ($P<0.05$) incidence in control group D (25%) than either of the vaccinated groups A, B and C (17.5%, 5% and 10% respectively) (Table 1). When reduction in the incidence of mastitis was compared among vaccinated and control cattle groups, it was significantly lower in group B (80%) followed by group C (60%) and group A (30%) (Table 2).

Post challenge highest somatic cell count was recorded on day 10 for unvaccinated cattle group D (11.733±1.22), whereas it was at peak at day 8 in case of group A and C (6.442±1.39 and 4.088±0.09) while highest post challenge somatic cell count was observed at day 4 (3.887±1.11) for group B (Fig. 11). There was a significant decrease in post challenge somatic cell count in all the vaccinated groups as compared to control group. The lowest overall mean somatic cell count was recorded in group B (2.323±0.46) followed by group C (3.006±0.43), group A (3.759±0.82) and unvaccinated group D (7.798±1.11) (Fig. 12).

DNCB results demonstrated that, after 24 hours, the skin thickness of cattle in group B vaccinated with MMRSAV (10.4 mm) increased the most, followed by cattle in group C vaccinated with AMRSAV (9.7 mm) and group A vaccinated with PMRSAV (8.4 mm). The skin thickness of the unvaccinated cattle group D was the thinnest (7.3 mm). After 48 hours, a similar decreasing trend was found in skin thickness; with the B group having the smallest reduction (9.8mm), followed by the C and A groups (8.6mm and 7mm respectively). The unvaccinated control group D, on the other hand, had the least thickness after 48 hours (5.7mm) (Fig. 13).

DISCUSSION

Staphylococcus aureus (*S. aureus*) considered as one of the most important udder pathogens causing significant economic losses worldwide (Boss *et al.,* 2016) has 20.35% mean prevalence in the world whereas Pakistan observes 52.3% (Sarwar 2013) and this pathogen has

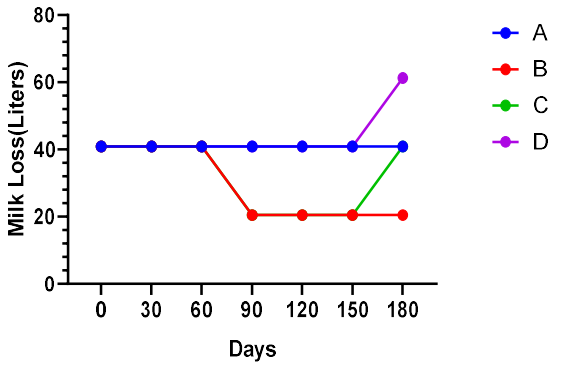

Fig. 10: Estimated milk loss of cattle groups during six months using LSSCS method.

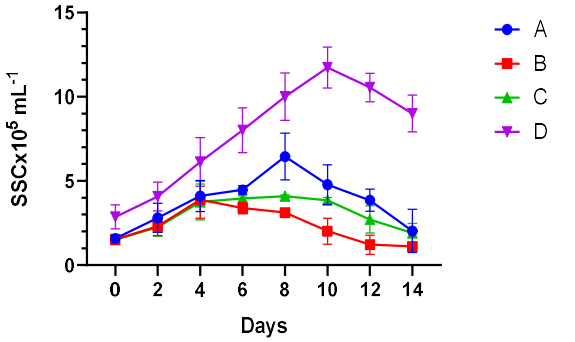

Fig. 11: Post challenge somatic cell count of cattle groups against MDRSA vaccines.

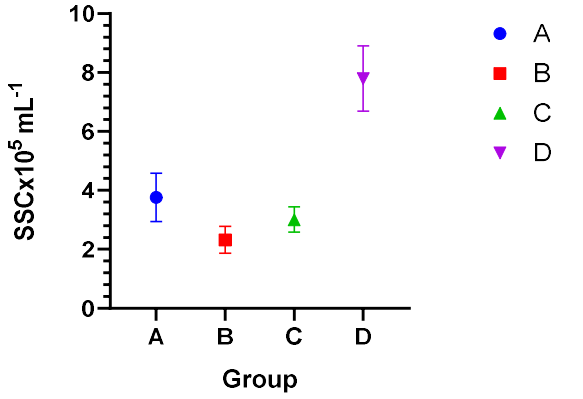

Fig. 12: Post Challenge mean somatic cell count for different groups.

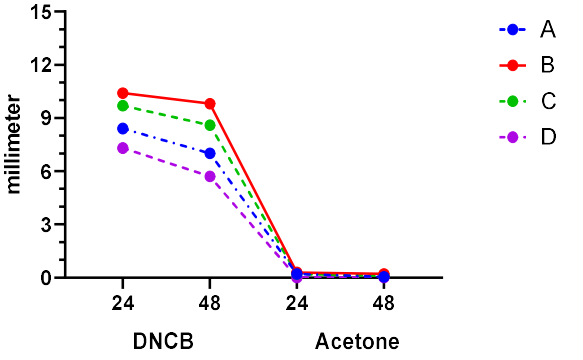

Fig. 13: Skin thickness of cattle groups in response to DNCB test.

Table 2: Percent reduction incidence as compared to control group

Group	Vaccine	*Incidence %	**Control group%	Reduction %
A	PMRSAV	17.5	25	30
B	MMRSAV	5	25	80
C	AMRSAV	10	25	60

PMRSAV= *Plain multidrug resistant S. aureus vaccine,* MMRSAV= *Montanide adjuvanted multidrug resistant S. aureus vaccine,* AMRSAV= *Aluminum hydroxide adjuvanted multidrug resistant S. aureus vaccine.*

been found 38 to 80% resistant to antibacterials (Devriese *et al.* 1997). Mastitis treatment with antibiotics is extremely difficult to cure, and drug residues have become a public health concern. Multiple approaches are needed to prevent infections and reduce the use of antimicrobial drugs. Among these, vaccination is viewed as a viable alternative to antibiotics. Vaccines are effective tools for preventing infections, and they have the potential to control and prevent AMR (Scali *et al.,* 2015). Researchers have been working on *S. aureus* mastitis vaccine for the past four decades, but the results have been inconsistent and variable. To be effective, a vaccination must sustain serum and milk whey antibody titers for a specified period of time (Athar, 2007). In present study, indirect haemagglutination assay (IHA) was used for estimation of serum and whey antibody titer. The test has been reported have sensitivity and specificity (62 and 96%) comparable to that of ELISA (sensitivity 69% and 90% specificity) technique (Dhanalakshmi *et al.,* 2016).

MMRSAV injected to cattle group B had the highest serum IHA antibody titer, followed by AMRSAV, PMRSAV administered to cattle group C and A. This is in agreement with the findings of Yousaf *et al.* (2009). Similar findings of were observed by Tollersrud *et al.* (2002) after vaccinating ewe with Montanide adjuvanted bacterial toxoid. The present study is in line with the findings of Giraudo *et al.* (1997) but in partial agreement with those of Shakoor (2006) and Athar (2007), reason being use of different adjuvant in previous studies where peak antibodies produced two months post vaccination whereas in present study highest serum antibody titer was observed at day 90 for Montanide oil adjuvanted vaccine and maintained higher for six month duration as compared to previous studies.

MMRSAV (Montanide oil adjuvanted vaccine) vaccinated cattle group B developed more milk whey antibodies than the other groups in the study. However, milk whey antibody titers were lower than serum antibody titers in all of the groups. This could be because blood immunoglobulins do not freely enter mammary glands. In previous studies researchers (Nordhaug *et al.,* 1994b; Shakoor 2006; Athar 2007; Yousaf *et al.,* 2009) also reported same findings of lower milk whey antibody concentration in systemic vaccination.

The somatic cell count, which is used as a marker for detecting subclinical mastitis, is critical to the health of the mammary gland. These are leukocytes (mostly macrophages, lymphocytes, and neutrophils) that are triggered by lymphokines in response to bacterial invasion in the mammary glands, resulting in an influx of neutrophils into the mammary gland, functioning as the principal resistance factor against invading pathogens. In present study, somatic cell count was recorded significantly low ($P<0.05$) in vaccinated groups as compared to control group. The present study findings are in line with those of (Ruegg 2001; Butt 2006; Shakoor 2006; Athar 2007; Yousaf *et al.,* 2009). In contrast to these findings, Giraudo *et al.* (1997) and Pellegrino *et al.* (2008) observed that vaccine did not affect somatic cell count significantly. This contradiction might be due alterations in vaccine composition, administration route, sampling time, stage of lactation of vaccinated animals.

The cattle vaccinated with adjuvanted vaccines resulted in significant higher milk yield in six-month duration than the control group. Among vaccinated groups the cattle injected with MMRSAV (Montanide oil adjuvanted vaccine) produced more milk as compared to cattle group injected with AMRSAV (Aluminum hydroxide adjuvanted vaccine). The increase in milk yield in vaccinated group are in accordance with the findings of (Leitner *et al.,* 2003; Pellegrino *et al.,* 2008). Similar findings were also reported previously (Butt, 2006; Shakoor, 2006; Athar, 2007; Yousaf *et al.,* 2009). However, (Watson, 1984) noticed a drop in milk output the day after vaccination, which was thought to be attributable to vaccination stress. Milk yield was measured on a monthly basis in this study, which reduced the stress component of vaccination, resulting in a positive impact on milk production.

Increased somatic cell count and milk yield in lactation animals have an inverse connection (Reneau, 1986). In the current study, the control group experienced a considerable milk loss of 306 liters as compared to the vaccinated groups. Milk loss was higher in group A vaccinated with plain vaccine (PMRSAV) compared to group B and group C cattle vaccinated with adjuvanted vaccines; however, milk loss in group A was lower than control. The vaccinated cattle groups, on the other hand, produced more milk than the control group when compared on an additional milk production basis. These findings are in agreement with (Athar, 2007).

Over the course of six months, the vaccinated cattle groups had a much lower quarter based incidence than the control group. Considering incidence rate among vaccinated cattle, there was a substantial difference amongst adjuvanted and plain vaccinated cattle groups. These findings are consistent with those of Tollersrud *et al.* (2002), Athar (2007) and Yousaf *et al.* (2009) who also found that vaccinated animals had fewer mastitic quarters. Other researchers (Nordhaug *et al.,* 1994a; Giraudo *et al.,* 1997; Shakoor, 2006) also found that vaccinated animals had reduced cumulative point incidence.

In cattle injected with adjuvanted vaccinations, the post-challenge somatic cell count was considerably lower than in cattle vaccinated with plain vaccines and the control group. The elevated somatic cell count in the control group was ascribed to protection failure, whereas a temporary rise in somatic cell count was observed in the vaccinated group. This rise in vaccinated individuals could be attributed to a rapid invasion of protective antibodies into the udder. The current study's conclusions are consistent with previous research (Shakoor, 2006; Athar, 2007; Yousaf *et al.,* 2009). However, these findings contradict those of Pellegrino *et al.* (2008), who found no difference in somatic cell count between

vaccinated and control cattle after challenge. This discrepancy could be caused by a difference in the challenge process or the strain selected for the challenge.

Cell-mediated immunity plays a vital role in host defense against pathogens. After vaccination, it also develops resistance to infections. Several intra-cutaneous and contact sensitivity tests have been developed to evaluate this sort of immunity. Humans commonly employ the 2, 4-dinitrochlorobenzene contact sensitivity test. In response to some of DNCB's metabolites, direct contact with it causes systemic sensitization. It creates hapten molecules once it comes into contact with the skin. A 5mm increase in diameter is an acknowledged criterion for delayed hypersensitivity in humans but no such criteria exist for animals. Skin fold thickness in vaccinated cattle was 8.4, 10.4, 9.7 mm after 24 hours post challenge in the A, B, and C groups respectively which decreased to 7, 9.8 and 8.6 mm after 48 hours indicating that the vaccinated groups had more thickness than the control group. These findings are consistent with those of Shakoor (2006), Athar (2007) and Yousaf *et al.* (2009) who found that vaccinated groups had thicker skin folds than control groups.

Conclusion: Montanide adjuvanted MDR *S. aureus* vaccine (MMRSAV) provided persistent and higher antibody titer than Aluminum hydroxide adjuvanted MDR *S. aureus* (AMRSAV) and plain MDR *S. aureus* vaccine (PMRSAV) that persisted at higher level for the entire study period which is correlated with slow release of vaccine from depots that are made by oil droplets**.** So, on the basis of the results of the current study, it was concluded that Montanide adjuvanted MDRSA vaccine was most effective in terms of increased persistent serum and milk whey antibody titers, decreased SSC, increased milk yield, decreased milk loss and decreased mastitis incidence thus can reduce MDR *S. aureus* mastitis in dairy cattle, new IMI infections and prevent antimicrobial resistance.

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