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Vaccines for Bovine Mastitis are Safe and Efficacious in Laboratory Animals

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ABSTRACT

Mastitis is the most costly disease of dairy cattle and poses colossal economic losses to the farming community worldwide. The role of vaccination in controlling mastitis is of paramount importance and significantly reduces the use of antimicrobial agents. This study aimed at preparing and evaluating inactivated polyvalent mastitis vaccines containing bovine origin *Staphylococcus aureus* (*tst*), *Streptococcus uberis* (*cpn-60* targeted *STUB*), and *Escherichia coli* (*aggR*) in a rabbit model. Four types of mastitis vaccines viz. formalin-inactivated plain polyvalent mastitis vaccine (FPPV), formalin-inactivated montanide adjuvanted polyvalent mastitis vaccine (FMPV), formalin-inactivated aluminum hydroxide adjuvanted polyvalent mastitis vaccine (FAPV), and formalin-inactivated montanide plus aluminum hydroxide adjuvanted mastitis vaccine (FMAPV) were prepared after confirmation for antigenicity and immunogenicity and evaluated for safety, side effects, challenge protection assay, sterility and humoral response in laboratory animals. Safety test showed no general adverse reactions to the vaccines when injected either into mice or rabbits or cow calves. Challenge protection studies revealed a significantly higher survival rate in vaccinated mice and rabbits compared to placebo groups. None of the vaccines when streaked onto culture media showed any growth indicating sterility of the vaccines. Repeated measures ANOVA was applied to compare mean O.D values of treatment groups for evaluation of humoral immunity. Serum ELISA O.D values against *S. aureus (tst), Str. uberis (cpn-60 STUB)* and *E. coli (aggR)* in vaccinated groups randomly inoculated i/m at thigh region @ 0.2 ml per rabbit were significantly higher ($p<0.05$) compared to control. Likewise, the humoral immune response of FAPV was highest followed in order by FMPV, FMAPV, and FPPV. It was concluded that newly-prepared polyvalent mastitis vaccines were safe to use, protective, and elicited a significant humoral immune response in rabbits.

INTRODUCTION

Pakistan has semi-arid land and subtropical climate with agriculture as the backbone of the country's economy. Pakistan is ranked as the 4th largest milk-producing country in the world having a 51.5 Million estimated cattle population with annual milk production of ~ 63.7 Million Tonnes. The livestock sector contributes ~ 60.1 percent to the agriculture value addition and ~ 11.5 percent to Pakistan's gross domestic product (Anonymous, 2021). In Pakistan, the livestock sector is composed mainly of small-holder dairy farmers possessing 2-3 domestic animals to fulfill their daily milk requirements. The milk beyond farmers' own requirement is sold and serves as a source of income for poor farming communities comprising mainly of women hence contributing to food security (Rehman *et al*., 2017).

The dairy industry in Pakistan is emerging and people are importing exotic cattle to establish corporate dairy farms. Despite the fact, Pakistan's dairy industry has a great export potential to generate $30 billion/annum but this industry needs many improvements to ensure the acceptability of its products in an international market.

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This industry can play a vital role to meet three out of seventeen United Nations Sustainable Development Goals (SDGs) related to poverty alleviation in all its forms everywhere, ensure food security, improved nutrition and promote sustainable agriculture to end hunger and ensure healthy lives and promote well-being for all at all ages with special reference to Pakistan. For this purpose, Pakistan Dairy Development Council (PDDC) has introduced many innovative measures. It is the feature of Pakistan's dairy industry that a prominent ratio of its labor force is from the neglected strata of society, the rural women. Furthermore, this study is an attempt towards achieving SDGs 3.B and 3.D, targeting the development of vaccines with affordable access for the communicable and non-communicable diseases primarily affecting developing countries to ensure public health, risk reduction, and management of national and global health risks (Hussain and Zaheer, 2020).

Mastitis has a significant global impact on the economics of the dairy industry (Ruegg, 2017). The annual economic loss due to mastitis was estimated to be US$200 per cow per year worldwide (Krishnamoorthy *et al*., 2021). The economic losses due to both clinical and subclinical mastitis (3-40 times more common than clinical) were USD 98,228 million annually in India, as reported in previous studies (Banal and Gupta, 2009). Ashfaq *et al*. (2015) have reported that mastitis costs 10.6 USD in terms of milk loss and USD 1.7 as treatment costs in dairy cows.

Major mastitogens routinely used to prepare mastitis vaccines are *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae,* and *Escherichia coli* (Rainard *et al*., 2021). In developing countries including Pakistan, no appropriate mastitis control programs exist and an increasing trend of antibiotic resistance against mastitogens has been observed (Khan *et al*., 2018). Therefore, it seems workable and economical to produce vaccines against the most common mastitogens to reduce losses due to mastitis. There is a growing appreciation for the role of vaccines in confronting the problem of antimicrobial resistance (AMR) and reducing the emergence and spread of antimicrobial-resistant bacteria (Dego, 2020). Vaccines can reduce the development of antimicrobial resistance by reducing the need for antimicrobial use to treat mastitis. The significant use of antibiotics in food-producing animals (more than 50% of global antibiotic consumption) as well as the transmission of drug-resistant microbial species to humans is a global cause for concern (Chokshi *et al*., 2019). The irrational use of antibiotics to treat bovine mastitis is an important factor in the development of AMR and has become a global public health concern (Abdi *et al*., 2021).

Several studies in cows have investigated the efficacies of monovalent vaccines against mastitis caused by *S. aureus* (Watson *et al*., 1996), *S. uberis* (Leigh, 2002), and *E. coli* (Tomita *et al*., 2000). In view of the association of a variety of mastitogens and their antigenic diversity, many workers have considered a polyvalent vaccine conceivably more pragmatic (UK, 1990; Yancey, 1999). A number of studies have evaluated the role of polyvalent vaccines in the control of bovine mastitis (Giraudo *et al*., 1997; Petrik *et al*., 1978). Although a few reports on the evaluation of mastitis vaccines in buffaloes are available in Pakistan (Athar *et al*., 2007; Shakoor *et al*., 2006) but no reports on the development of a polyvalent vaccine for dairy cows are available in Pakistan. Since bovine mastitis in Pakistan is caused mainly (~84%, collectively) by *S. aureus, S. agalactiae,* and *E. coli*, the development of an effective vaccine for these three mastitogens is likely to significantly impact mastitis control in the country. Therefore, the present study was designed to evaluate the efficacy of a locally-prepared inactivated polyvalent mastitis vaccine containing *S. aureus* (*tst*), *S. uberis* (*cpn-60* targeted *STUB*), and *E. coli* (*aggR*) under laboratory and field conditions.

MATERIALS AND METHODS

Ethical approval

All samples were collected as per standard sample collection procedures by trained veterinarians without any discomfort to the animals. The present work was carried out following the guidelines of the Ethical Review Committee of the University of Veterinary and Animal Sciences (UVAS) Lahore, Pakistan.

Bacterial isolates

The most prevalent toxinotypes of *Staphylococcus aureus (tst)*, *Streptococcus uberis (chaperonin-60 targeted STUB),* and *Escherichia coli (aggR)* isolated from the milk of mastitic dairy cows were used for vaccines production. These isolates were obtained from Animal Health Research Laboratory, Department of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore, Pakistan. The procedures for isolation of said bacterial isolates are already described by Chaudhary and Azam (1995) and Gonzalez *et al*. (1990). The morphological and biochemical determination of the isolates was done as per the previous studies conducted by El-Jakee *et al*. (2008) and Raza *et al*. (2015).

Preparation of plain and adjuvanted polyvalent mastitis vaccines

Optimization of cultural conditions was done to

express pseudocapsule of *S. aureus* (*tst*) and to provide growth factors to *S. uberis* (*cpn-60 STUB*), and *E. coli* (*aggR*). The optimum growth conditions were provided to *S. aureus (tst)* in order to get encapsulated bacteria (Watson and Watson, 1989). For this purpose, the selected field isolate was grown on modified nutrient broth. Modification of nutrient broth was done by incorporating sterile bovine whey at 10% v/v as previously described (Athar *et al*., 2007). The extent of expression of pseudocapsule was confirmed by using the autoagglutination method (Ahmad and Muhammad, 2008; Aqib *et al*., 2018; Athar *et al*., 2007; Watson and Watson, 1989). Sterile bovine whey (10% v/v) was also added to the broth media for *S. uberis (cpn-60 STUB),* and *E. coli (aggR)* to enhance their growth (Brown and Baetz, 1976).

For the preparation of FPPV, the said bacterial isolates were grown independently, in modified nutrient broth at 37°C for 48 h on an orbital shaker (MaXshake, DAIHAN Scientific North America Inc. Largo, FL, USA) set at 60 rpm under aerobic conditions. After confirming the purity and presence of pseudocapsule of *S. aureus*, the suspension was inactivated with formalin (0.4% v/v) (Cheng and Han, 2020), centrifuged at 6000 x*g* for 60 min at 4°C. The supernatant was collected, autoclaved, and stored at 4°C for further use as a toxoid. The bacterial isolates containing pellet was resuspended in phosphate-buffered saline (pH 7.2) by using a pH meter (Thermo Fisher Scientific, Waltham, MA, USA). These suspensions were preserved at 4°C until required. Bacterial concentrations were adjusted to 1 x 10^{10} cells / mL for each of the *S. aureus (tst), S. uberis (cpn-60 STUB),* and *E. coli (aggR)* spectrophotometrically (Hirsch and Strauss, 1964). For incorporation of crude toxin extract, supernatant fluids were collected from a 48-h broth culture of *S. aureus (tst)* and *S. uberis (cpn-60 STUB)*. The supernatants were autoclaved and centrifuged at 6000 x*g* for 30 min at 4°C and supernatants were added in vaccine preparation at concentration ~5 mg of dry weight per dose (Giraudo *et al*., 1997). Sodium azide, thimerosal, and formalin were added as preservatives at final concentrations of 0.001%, 0.001%, and 0.4%, respectively (Supplementary Table I).

For the preparation of the FMPV, the formalin-inactivated plain polyvalent vaccine was prepared as described above and montanide was added to it as an adjuvant. Briefly, one liter of montanide was homogenized with an equal volume of antigenic preparation drop by drop during homogenization using Ultratourex (HG-15D, Homogenizer, DAIHAN Scientific North America Inc., Largo, FL, US) (Supplementary Table I).

For the preparation of the FAPV, the formalin-inactivated plain polyvalent vaccine was prepared as described above and aluminum hydroxide was added to it as an adjuvant. Briefly, aluminum hydroxide gel was prepared and added to the formalin-inactivated plain polyvalent vaccine at 3.5% (Giraudo *et al*., 1997) (Supplementary Table I).

For the preparation of the FMAPV, the formalin-inactivated plain polyvalent vaccine was prepared as described above and both montanide and aluminum hydroxide were added in the same concentrations as described above for vaccines individually adjuvanted with them (Supplementary Table I).

For the preparation of Placebo, phosphate-buffered saline (PBS; pH 7.2) supplemented with sodium azide and thimerosal at the same concentrations as used in the vaccine was used as a placebo (Supplementary Table I).

Evaluation of sterility of vaccines

The sterility of vaccines was evaluated by streaking of a loopful of the vaccine on blood and MacConkey's agar plates and was incubated for 48 h. No bacterial growth was indicative of the sterility of the vaccines (Aqib *et al*., 2018).

Determination of safety of vaccines in mice, rabbits, and cow calves

Samples of prepared vaccines were evaluated for safety, side effects, and immunogenicity against *S. aureus (tst), S. uberis (cpn-60 STUB),* and *E. coli (aggR)* in mice (Giraudo *et al*., 1997). Thirty, mice and rabbits each while 20 cow calves were divided into 5 groups of 6, mice and rabbits each while 4 calves each (group A-D, vaccinated while E designated as control) injected 1 mL of each vaccine to group A-D mice and group E (placebo) intraperitoneally.

Half of the rabbits in each group (3 rabbits) were injected 0.2 mL of vaccine subcutaneously (SC) and another half of the rabbits were given 0.5 mL of vaccine SC.

Subcutaneous and intramuscular administration of 2 doses (2mL) of vaccine (one on either side of the neck) was performed in group A-D calves and placebo in group E calves. Any local or systemic reaction and mortality were observed for up to 7 days in all the vaccinated animals mentioned above. This safety trial was conducted as a preliminary study to be replicated in candidate animal i.e. dairy cow.

Challenge study in mice and rabbits

A total of 60 mice were divided into 5 groups (A-E) comprising of 12 mice in each group. Rabbits in each group were injected intraperitoneally with two (0.1mL) doses of the vaccine at 15 days intervals. After 30 days of the administration of 2nd dose of vaccine, mice in each

group were challenged with an inoculum containing 10^{10} cells/mL of each of *S. aureus* (*tst*), *S. uberis* (STUB), and *E. coli* (*aggR*). The number of dead mice within 3 days of the challenge were recorded and compared to calculate the percent survival rate.

For this purpose, thirty adult healthy rabbits were randomly divided into 5 groups of 6 (A-E). Rabbits in four groups (A-D) were vaccinated intramuscularly twice with a 0.2 mL dose of each experimental vaccine at 15 days intervals. While rabbits in group E were given a placebo at the same dose rate as a vaccine. On day-30, after booster vaccination, rabbits in all groups were challenged with 1mL of live inoculum (10^{10} cells/mL) of the 3 vaccinal bacterial isolates. Mortality in all groups was recorded till day-7 post-challenge for calculating percent survival.

Evaluation of humoral immunity in rabbits

A total of 30 adult healthy male rabbits were divided into 5 groups (A-E) with 6 rabbits in each group. Rabbits in group A were vaccinated with formalin-inactivated plain polyvalent vaccine and rabbits in group B were administered formalin-inactivated montanide adjuvanted polyvalent vaccine through intramuscular route at the dose rate of 0.2 ml per rabbit at thigh region. The rabbits in group C were injected formalin-inactivated aluminum hydroxide-adjuvanted vaccine while the rabbits in group D received formalin-inactivated montanide plus aluminum hydroxide-adjuvanted vaccine at the same dose rate via the same route. The placebo was administered at the same dose rate via the same route to the rabbits in group E that served as control. Booster vaccine dose was given on day 15 of the first injection. The rabbits were kept for two month for post-vaccination evaluation of serum samples and were exercised fortnightly. Serum antibody titers against each of the vaccinal isolates were measured at days 0, 15, 30, 45, and 60 post-vaccination by a commercially available ELISA kit (Abbexa, Cambridge, United Kingdom). In the present study, ELISA mastitis kit was used for the detection of humoral immune response in rabbits injected with toxinotyping-based polyvalent mastitits vaccine whereas previous studies in Pakistan used Indirect Haemagglutination (Aqib *et al*., 2018; Raza *et al*., 2015). ELISA mastitis kit was used for detection of humoral immune response in rabbits injected with toxinotyping based polyvalent mastitis vaccine whereas all previous studies used Indirect Haemagglutination Assay (Aqib *et al*., 2018; Raza *et al*., 2015).

Statistical analysis

The data originating from different studies were subjected to repeated measures analysis of variance (ANOVA) with the significance level set at 5% using SAS software (version 9.1) to ascertain the immune response to polyvalent mastitis vaccine in the treatment and control groups. For the comparison of significant treatment mean Duncan's multiple range test was applied.

RESULTS

Sterility of vaccines

None of the vaccines when cultured on blood and MacConkey's agar plates showed any growth in 48 h which indicated the sterility of the vaccines under trial (Aqib *et al*., 2018).

Safety of vaccines in mice, rabbits, and cow calves

None of the mice died following intraperitoneal (IP) administration of 1mL in any of the 4 experimental vaccines group as well as a control group. A 25/30 (83.33%) mice showed normal behavior, while 5 mice showed slight behavioral changes (16.66%). Out of 5 having abnormal behavior, 3 of the mice had bristled hairs with sluggish movement whereas 2 mice were observed lethargic during the first 24 h after injection. The safety trials of 4 experimental vaccines in mice suggest that the tested vaccines had no adverse effects (Table I)

Table I. Safety and side effects of polyvalent mastitis vaccines in mice.

Vaccine type	n	Normal behavior	Lethargic + Listless for		
			6 h	12 h	24 h
FPPV	6	5	1	-	-
FMPV	6	5	-	1	-
FAPV	6	5	-	-	-
FMAPV	6	4	-	-	2
Placebo	6	6	-	-	-

FPPV, Formalin-inactivated plain polyvalent mastitis vaccine; FMPV, Formalin-inactivated montanide adjuvanted polyvalent mastitis vaccine; FAPV, Formalin-inactivated aluminum hydroxide adjuvanted polyvalent mastitis vaccine; FMAPV, Formalin-inactivated montanide plus aluminum hydroxide adjuvanted polyvalent mastitis vaccine. Dose and route of vaccine = 0.1 mL I.P; No mortality was recorded in all 30 mice.

The results of the comparative safety of vaccines in rabbits are presented in (Table II). After subcutaneous (SC) administration of the two doses (0.2 or 0.5mL) of all the 4 vaccines in rabbits, neither swelling at the site of injection nor any other untoward effects were noticed. In group B, with a higher dose (0.5mL) of montanide-adjuvanted vaccine, 1/3 rabbits showed slight lethargy. In group D of montanide plus aluminum hydroxide-adjuvanted group,

2/3 rabbits, who got higher dose 0.5mL SC, showed prickled hairs and lethargy. None of the rabbits died following SC administration of 0.2mL and 0.5mL in any of the 4 experimental vaccine groups or the control group. The results of the safety trials of 4 experimental vaccines in rabbits suggested that they are 100% safe with no adverse effects when administered in rabbits at the mentioned dose rate (Table II).

Table II. Safety and side effects of polyvalent mastitis vaccines in rabbits.

Vaccine type	n	Dose and route	No. of rabbits	Behavior change	
				No.	Behavior
FPPV	6	0.2mL SC	3	3	Normal
		0.5mL SC	3	3	Normal
FMPV	6	0.2mL SC	3	3	Normal
		0.5mL SC	3	1	Slight lethargic movement
FAPV	6	0.2mL SC	3	3	Normal
		0.5mL SC	3	3	Normal
FMAPV	6	0.2mL SC	3	3	Normal
		0.5mL SC	3	2	Prickled hairs and lethargic movement
Placebo	6	0.2mL SC	3	3	Normal
		0.5mL SC	3	3	Normal

SC, subcutaneous; FPPV, Formalin-inactivated plain polyvalent mastitis vaccine; FMPV, Formalin-inactivated montanide adjuvanted polyvalent mastitis vaccine; FAPV, Formalin-inactivated aluminum hydroxide adjuvanted polyvalent mastitis vaccine; FMAPV, Formalin-inactivated montanide plus aluminum hydroxide adjuvanted polyvalent mastitis vaccine. No injection site swelling and mortality were recorded in all 30 rabbits.

A safety study of 4 experimental vaccines in cow calves also clearly demonstrated that the vaccines are 100% safe with no adverse effects as 1/4 calves in group B and D in which 1mL SC Montanide and Montanide plus aluminum hydroxide vaccines were injected, respectively developed mild to moderate swelling at the injection site. The swelling subsided within 48 h whereas intramuscular (IM) administration of all types of vaccines showed no swelling. None of the calves administered with subcutaneous and intramuscular administration of 2 doses (2mL) of vaccine (1mL on either side of neck) in any of 4 experimental vaccine groups as well as control group died. The data suggest that the vaccines are safe to use in cows (Table III).

Table III. Safety and side effects of polyvalent mastitis vaccines in cow calves.

Type of vaccine	n	Dose/ Route	Swelling at injection site		Fever	Mortality
			No.	Pattern		
FPPV	4	1mL SC	4	None	None	0
		1mL IM	4	None		0
FMPV	4	1mL SC	1	Mild/moderate	None	0
		1mL IM	4	None		0
FAPV	4	1mL SC	4	None	None	0
		1mL IM	4	None		0
FMAPV	4	1mL SC	1	Mild/moderate	None	0
		1mL IM	4	None		0
Placebo	4	1mL SC	4	None	None	0
		1mL IM	4	None		0

Mild/Moderate: Swelling at the injection site which subsided within 24 h. SC, subcutaneous; IM, intramuscular.

Challenge protection in mice and rabbits

None of the mice from groups C and D (vaccinated twice before challenging) died within 3 days (survival rate 100%) of the challenging IP with 1 mL of live inoculum containing 10^{10} cells/mL of each of the vaccinal isolates whereas only one mouse survived from the control group (which also died later on day 5). The survival rate in mice of Group D, C, B, A, and E was measured as 100, 100, 91.7, 83.3, 8.3%, respectively (Table IV).

A similar trend was observed in vaccinated and control rabbits where vaccinated rabbits had 83.3-100% survival as compared to the control group where only 16.7% of the rabbits survived 7 days post-challenge. The said trials suggested that all the polyvalent vaccines are protective for the subject mastitogens (Table V).

Table IV. Challenge protection assay of polyvalent mastitis vaccines in mice.

Type of vaccine	n	Challenge dose and route at day-30 post-booster dose	No. of mice died within 3 days	Survival rate (%)
FPPV	12	1mL IP	02	83.3
FMPV	12	1mL IP	01	91.7
FAPV	12	1mL IP	0	100
FMAPV	12	1mL IP	0	100
Placebo	12	1mL IP	11	8.3

IP, Intraperitoneal. Vaccine dose injected at day-0 = 0.1mL IP; Booster dose injected at day-15 = 0.1mL IP.

Table V. Challenge protection assay of polyvalent mastitis vaccines in rabbits.

Type of vaccine	n	Challenge dose and route at day-30 post-booster dose	No. of rabbits died within 7 days	Survival rate (%)
FPPV	6	1mL IM	01	83.3
FMPV	6	1mL IM	0	100
FAPV	6	1mL IM	0	100
FMAPV	6	1mL IM	0	100
Placebo	6	1mL IM	05	16.7

IM, Intramuscular; Dose of vaccine injected at day-0 = 0.2mL IP; Booster dose at day-15 = 0.2mL IM.

Humoral immune response of polyvalent mastitis vaccines in rabbits

Serum ELISA OD values against *S. aureus* (*tst*) were the highest (3.19±0.12) at day-30 in rabbits of formalin-inactivated aluminum hydroxide-adjuvanted polyvalent vaccine: FAPV (group C) followed by a steady decrease at day 45 and day 60. This was followed by rabbits of formalin-inactivated Montanide oil-adjuvanted polyvalent vaccine: FMPV (group B) where the highest titer (3.07±0.09) was achieved at day-30 followed by a steady decrease at day 45 and day 60. Rabbits in formalin-inactivated montanide oil plus aluminum hydroxide-adjuvanted polyvalent vaccine (group D) were the next, where serum ELISA antibody titer peak value (2.91±0.12) was at day 30 followed by a slight decrease at day 45 but significant decrease (2.41±0.13; $p<0.05$) at day 60. By following group C, B, and D, formalin-inactivated plain polyvalent vaccine (group A) peak ELISA OD value was 2.90±0.03 at day-30 followed by a slight decrease at day 45 but a significant decrease (2.35±0.03; $p<0.05$) at day 60. All the groups: A, B, C, and D when compared with the control group (E) demonstrated statistically higher OD values ($p<0.05$). Similarly, the immune response generated at different days: 15, 30, 45, and 60 when compared with day 0 revealed statistically higher antibody levels ($p<0.05$) (Fig. 1A).

For *S. uberis* (STUB), ELISA antibody titer was the highest (3.02±0.09) at day-30 in rabbits of formalin-inactivated aluminum hydroxide-adjuvanted polyvalent vaccine: FAPV (group C) followed by a steady decrease at day 45 and day 60. This was followed by rabbits of (group B) formalin-inactivated montanide oil-adjuvanted polyvalent vaccine: FMPV, where the highest titer (2.89±0.10) was achieved at day-30 followed by a steady decrease at day 45 but a significant decrease (2.38±0.03; $p<0.05$) at day 60. Likewise, rabbits in (group D) formalin-inactivated montanide oil plus aluminum hydroxide-adjuvanted polyvalent vaccine (FMAPV) showed a higher ELISA OD value (2.91±0.12) at day 30 followed by a slight decrease at day 45 but a significant decrease (2.30±0.08; P<0.05) at day 60. On the other hand, rabbits in (group A) formalin-inactivated plain polyvalent vaccine: FPPV had peak ELISA OD value (2.75±0.07) at day-30 followed by a slight decrease at day 45 and a significant decrease (2.26±0.06; $p<0.05$) at day 60. Overall, ELISA OD values at different days 15, 30, 45, and 60 when compared with day 0 revealed the statistically higher values ($p<0.05$) (Fig. 1B).

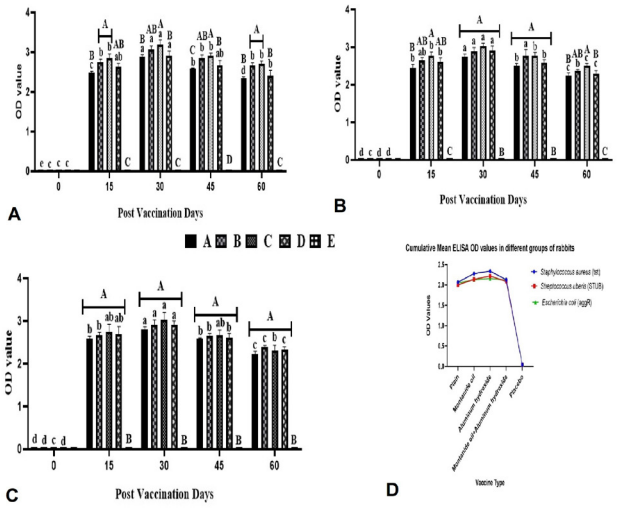

Fig. 1. ELISA OD values against *S. aureus (tst)* (A), *Strept. uberis (cpn-60 STUB)* (B), *E. coli (aggR)* (C), and cumulative mean ELISA OD values against *S. aureus (tst), Strept. uberis (cpn-60 STUB)* and *E. coli (aggR)* (D).

Similarly, the highest ELISA OD value (3.02±0.18) against *E. coli* (*aggR*) was observed in rabbits of (group C) formalin-inactivated aluminum hydroxide-adjuvanted polyvalent vaccine: FAPV on day-30 followed by a significant decrease ($p<0.05$) at day-45 and day-60. The same trend was observed in rabbits of group B (formalin-inactivated montanide oil-adjuvanted polyvalent vaccine), group D (formalin-inactivated montanide oil plus aluminum hydroxide-adjuvanted polyvalent vaccine) and group A (formalin-inactivated plain polyvalent vaccine). ELISA OD values against *E. coli* (*aggR*) at different days 15, 30, 45, and 60 when compared with day-0 revealed the statistically significant difference ($p<0.05$) (Fig. 1C).

When comparison was made on the basis of cumulative mean ELISA OD values, values were significantly higher ($p<0.05$) in rabbits of group A (formalin inactivated plain polyvalent vaccine), B (formalin inactivated montanide oil-adjuvanted polyvalent vaccine), C (formalin inactivated

aluminum hydroxide-adjuvanted polyvalent vaccine) and D (formalin inactivated montanide oil plus aluminum hydroxide-adjuvanted polyvalent vaccine) compared to group E (placebo). On the other hand, cumulative mean OD values of groups B and C were higher than OD values of rabbits in groups A and D (Fig. 1D).

DISCUSSION

Bovine mastitis is the most frequent issue faced by the dairy industry of Pakistan (Jokhio *et al*., 2021; Ruk *et al*., 2021). The control of mastitis in cows has become a challenge for dairy scientists especially in the wake of the emergence and spread of antimicrobial-resistant mastitogens and the zoonotic nature of some mastitogens (Abdi *et al*., 2021; Hussain and Zaheer, 2020). Polyvalent vaccines have proved to be efficacious against a variety of mastitogens. Moreover, the use of vaccines does not contribute to antimicrobial resistance (Dego, 2020). Therefore, the present study investigated different newly prepared killed polyvalent mastitis vaccines having the most prevalent mastitogens (*S. aureus* (*tst*), *S. uberis* (*STUB*), and *E. coli* (*aggR*)). These vaccines were effective in inducing a protective immune response in laboratory animals. The immune response was measured following quality control standards by using a commercially available ELISA kit (Abbexa, Cambridge, United Kingdom) suggesting vaccines could be an alternative to antimicrobials for the control of bovine mastitis in Pakistan.

The ELISA has emerged as an advanced, sensitive, and quick diagnostic and monitoring tool for the detection of antibodies generated in vaccinated animals (Kotb *et al*., 2021). Four different types of killed polyvalent mastitis vaccines have different adjuvants: Formalin-inactivated plain polyvalent mastitis vaccine (FPPV), formalin-inactivated montanide adjuvanted polyvalent mastitis vaccine (FMPV), formalin-inactivated aluminum hydroxide adjuvanted polyvalent mastitis vaccine (FAPV), and formalin-inactivated montanide plus aluminum hydroxide adjuvanted mastitis vaccine (FMAPV) were prepared and evaluated in laboratory animals. Statistically higher serum ELISA antibody titers ($p<0.05$) were recorded in all four vaccine groups (A, B, C, and D) compared to the control group (E). Similarly, the immune response generated at different days: 15, 30, 45, and 60 when compared with day 0 revealed the statistically higher ($p<0.05$) antibody response.

For this purpose, sourcing of isolates and development of different vaccine candidates with different adjuvants were carried out following quality measures (sterility, safety, side effects) and assessment of different vaccine candidates dose safety (challenge-protection assay) and efficacy of humoral immunity in laboratory animals.

The results of the sterility evaluation revealed that the newly-prepared polyvalent mastitis vaccines were free from any extraneous contaminants (aerobic and anaerobic bacteria, mycoplasma, and fungi suggesting the vaccine were sterile (Aqib *et al*., 2018).

No general adverse reactions or untoward effects to the newly-prepared polyvalent mastitis vaccines were observed in vaccinated mice, rabbits, and calves at the mentioned dose rates throughout the safety trial period suggesting that the vaccines are safe to use corroborating Aqib *et al*. (2018).

The challenge-protection assay revealed a significantly higher survival rate both in vaccinated mice and rabbits compared to the control mice and rabbits. These findings are consistent with previous studies (Ahmad and Muhammad 2008; Giraudo *et al*., 1997; Perez *et al*., 2009; Raza *et al*., 2015) and suggest that the tested polyvalent vaccines are protective for the subject mastitogens.

Inactivated whole organism vaccines generally require the addition of an adjuvant as an immune-potentiator. Adjuvants play an important role in stimulating the protective efficacy of a number of different vaccines (Fleck *et al*., 2006). In the present study, montanide oil, aluminum hydroxide, and montanide oil plus aluminum hydroxide were used as adjuvants to prepare polyvalent mastitis vaccines containing inactivated antigens along with crude toxin extract of subject mastitogens isolated from mastitic milk.

Serum ELISA OD values against *S. aureus* (*tst*) in all the vaccinated groups: A, B, C, and D demonstrated statistically higher OD values ($p<0.05$) in rabbits when compared to the control group (E). Similarly, statistically higher antibody response ($p<0.05$) was generated at different days: 15, 30, 45, and 60 against *S. aureus (tst)* also demonstrated elevating trend from day-1 up to day-30 followed by a decline till the end of a trial at day-60 in all vaccinated rabbits when compared with day-0 (priming). These results are consistent with the previous studies (Ahmad and Muhammad, 2008; Aqib *et al*., 2018; Athar *et al*., 2007).

For *S. uberis (cpn-60 STUB)*, a similar trend was noticed in serum ELISA OD values in rabbits as depicted by *S. aureus (tst)*. These findings are in agreement with the findings of Calonzi *et al*. (2020) and Niazi (2021) who optimized and evaluated an *S. uberis* inactivated vaccine in laboratory animals. However, the present study's results suggesting that the *S. uberis* (*cpn-60 STUB*) mastitis vaccine is highly effective in laboratory animals are not in agreement with (Dego, 2021; Kerro *et al*., 2021). Prior to this study, there has been found no effective commercial

S. uberis vaccine to control bovine mastitis due to various reasons such as the lack of proper characterization of such vaccines under controlled experimental studies, lack of field-based studies in candidate animals, use of imported vaccinal isolates, antigenic diversity of different strains of vaccinal isolates, poor management practices, lack of understanding of humoral and cellular intramammary immunity and lack of understanding of virulence traits of S. uberis. Most of these shortcomings were addressed in the present study.

For *E. coli (aggR),* a similar trend was observed as noticed for *S. aureus* (*tst*) and *S. uberis (cpn-60 STUB)*. There is no study reported so far regarding immunogenicity trials of a vaccine against *E. coli (aggR)* isolate in a rabbit model using an ELISA kit. Therefore, the results were compared and found consistent with *S. aureus* and *S. uberis* vaccine trials in rabbits (Ahmad and Muhammad, 2008; Magas *et al*., 2013).

In the present study, when comparison was made on the basis of cumulative mean ELISA OD values, rabbits vaccinated with either of four vaccines or kept as control, the statistically highest OD values against *S. aureus (tst), S. uberis (cpn-60 STUB),* and *E. coli (aggR)* were recorded in those given the vaccine (FAPV) containing aluminum hydroxide as an adjuvant. These findings are consistent with Zhang *et al*. (2018) who evaluated three different adjuvants: Aluminum hydroxide, montanide oil (Seppic SA, Paris, France), and Freund's incomplete adjuvant for bovine polyvalent mastitis vaccine in mice and reported that aluminum hydroxide demonstrated the best adjuvant effects. Aluminum hydroxide adjuvants stimulate immunity in a number of ways: it makes particulate of soluble antigens, which augments uptake through phagocytosis by dendritic cells; it targets antigen to antigen-presenting cells (APCs) while enhancing antigen-presentation, as indicated by increased expression of MHC II-peptide complexes and increased activation of CD4 T cells; and it retains antigen at the site of injection, allowing time for recruitment of APCs through the release of cytokines and the induction of a local inflammatory reaction. It also has been suggested that a short-term depot is not necessary for the effect of aluminum adjuvants (HogenEsch *et al*., 2018).

Aluminum hydroxide-adjuvanted vaccine provides rapid immune response making these vaccines the choice therapy (Sears *et al*., 1990). Aluminum hydroxide adjuvanted vaccine's immunity is generally does not last for prolonged duration, however, in the present study; such vaccines induced the highest antibody titer that sustained for a longer duration. This is not in agreement with the finding of Aqib *et al*. (2018), who reported that the alum-adjuvant mastitis vaccine was found weaker in inducing immune response compared to an oil-adjuvant vaccine (Aqib *et al*., 2018). The aluminum hydroxide and montanide oil-adjuvanted mastitis vaccines immune response in a rabbit model needs further investigation. Oil adjuvanted vaccine gained the peak titer slowly, but the immunity also waned gradually. This may be attributable to the depot effect and slow release of mineral oil-based vaccine components from the site of inoculation (Nordhaug *et al*., 1994).

The present study suggested that formalin-inactivated aluminum hydroxide-adjuvanted *S. aureus (tst), S. uberis (cpn-60 STUB),* and *E. coli (aggR)* vaccines are the safe and efficacious in inducing protective humoral immune response against mastitogens in laboratory animals. These findings are consistent with multiple previous studies (Abubakar *et al*., 2007; Aqib *et al*., 2018; Kerro-Dego *et al*., 2021; Perez *et al*., 2009; Raza *et al*., 2015).

CONCLUSION

The *Staphylococcus aureus* (*tst*), *Streptococcus uberis* (*chaperonin-60 targeted STUB*), and *Escherichia coli* (*aggR*) local field isolates from cow mastitic milk were antigenic as well as immunogenic. Polyvalent mastitis vaccines can be safe and efficacious in controlling mastitis in animals. Formalin-inactivated aluminum hydroxide-adjuvanted polyvalent vaccine (FAPV) demonstrated the highest and sustained immune response followed in order by formalin-inactivated montanide oil-adjuvanted polyvalent vaccine (FMPV), formalin-inactivated montanide oil plus aluminum hydroxide-adjuvanted polyvalent vaccine (FMAPV), and formalin-inactivated plain polyvalent vaccine (FPPV). These promising results from all the four vaccines warrant trials in ruminants for the control of mastitis caused by *Staphylococcus aureus*, *Streptococcus uberis*, and *Escherichia coli*.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20211212031238

Statement of conflict of interest

The authors have declared no conflict of interest.

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