Journal of Animal & Plant Sciences, 33(3): 2023, Page: 526-533
ISSN (print): 1018-7081; ISSN (online): 2309-8694
https://doi.org/10.36899/JAPS.2023.3.0645

EVALUATION OF IMMUNOGENICITY AND DOSE DEPENDENT IMMUNE RESPONSE AGAINST COMMON BOVINE ORIGINATED MASTITOGENS IN RABBIT MODEL

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ABSTRACT

This study evaluated the immunogenicity and dose-dependent immune response of toxinotypes of bovine origin viz *Staphylococcus aureus (tst), Streptococcus uberis (*Cpn-60 targeted STUB*),* and *Escherichia coli (aggR)* in a rabbit model – a step forward towards an effective polyvalent mastitis vaccine. To evaluate the primary and secondary immune response (immunogenicity) against the concentration of 10^6 cells/mL of each preparation containing subject vaccinal isolates, 24 rabbits were divided randomly into 4 equal groups viz A, B, C and D. Each antigenic preparation was inoculated to the rabbits of group A to C @ 0.2 mL SC while group D served as control (placebo) at day 0 as priming dose and a booster dose at day 7 respectively. The serum antibodies titers were recorded in terms of Optical Density (OD) values at day 0 (pre-inoculation), 7, 14, 21 and 28 (post-inoculation) by iELISA Mastitis Kit (Abbexa, UK). The antibody response was significantly higher ($p<0.05$) in the post-booster samples at day-14 and day-21 for all the selected isolates. The highest primary antibody response (1.74 ± 0.14) as well as secondary immune response (2.04 ± 0.13) was shown by *Strept. uberis* (Cpn-60 STUB) in group B on day-14 followed in order by group C and A respectively. Likewise, dose-dependent immune response to composite antigens of 3 different antigenic concentrations of vaccinal isolates: 10^8 cells/mL; 10^{10} cells/mL; 10^{12} cells/mL of each of vaccinal isolates in 18 adult rabbits divided randomly into 3 groups of 6 (A, B and C) respectively for dose standardization was evaluated. Serum samples were collected at weekly intervals following completion of 2nd shot of inocula of each dose for 3 consecutive weeks. The rabbits of group B injected with 10^{10} cells mL^{-1} of *S. aureus (tst)* showed a significantly higher ($p<0.05$) serum iELISA O.D value ($2.97^{A, a}$ ± 0.10) followed in order by *E. coli (aggR)* (2.84^{a} ± 0.08) and *Str. uberis (cpn60 STUB)* (2.78^{a} ± 0.07) at day-14 post-inoculation. This study revealed the concentration of 10^{10} cells/mL of each subject mastitogens as a standard bacterial load for polyvalent mastitis vaccines with higher and sustained antigenicity in rabbits.

Keywords: Toxinotypes, polyvalent vaccines, antibody titer, rabbits

Published first online February 10, 2023 Published final June 18, 2023

INTRODUCTION

Agriculture sector is the backbone of Pakistan's economy and fortunately this sector recorded a remarkable growth of 4.40% during fiscal year 2021-22. It has also been reported that the livestock sector contributes 61.89% to the agriculture value addition and 14.04% to Pakistan's gross domestic product (Anonymous, 2022). Despite the fact, dairy industry is emerging in the country but it encounters many challenges. Control of bovine mastitis is one of the biggest challenges of the said industry.

Bovine mastitis is primarily caused by numerous mastitogens having devastating effects on dairy industry and public health globally in terms of decreased milk quantity and deteriorated milk quality (Ruegg, 2017; Dego, 2020). The dairy industry across globe costs ~ US$ 200 per cow per year on account of mastitis (Krishnamoorthy *et al.,* 2021). It has been reported that dairy industry loses 35 billion USD per annum worldwide (Sathiyabarathi *et al.*, 2016). In district Faisalabad, Punjab-Pakistan, it was estimated that mastitis costs PKR 1873/- in terms of milk loss and PKR 293/- as treatment loss in dairy cattle (Ashfaq *et al.* 2015).

Staphylococcus aureus, Streptococcus uberis, *Streptococcus agalactiae, Streptococcus dysgalactiae,* and *Escherichia coli* are the major mastitis causing bacteria which were routinely used to develop mastitis

vaccines (Rainard *et al.,* 2021). In developing countries like Pakistan, appropriate mastitis control program is practically not implemented that's why increasing trend of antimicrobial resistance against mastitogens have become a dilemma. Therefore, it seems workable and economical to produce a vaccine against the most common mastitogens to reduce economic losses due to mastitis. The immunity of the mammary gland either innate or acquired varies towards different bacteria (Katsafadou *et al.*, 2019) or in relation to their associated toxins (Zigo *et al.,* 2021). Vaccination with killed whole bacterial organisms or bacterial proteins (Bacterins) has been used as one of the effective measures to control mastitis (Sears, 2003). Due to growing antimicrobial resistance and public health concerns, the era of antimicrobials is over. Development and application of modern vaccine as a novel therapeutic approach to control bovine mastitis is a dire need of time during this post-antibiotic era (El-Sayed and Kamel, 2021). Efficacious vaccines will have to provoke immunity different from those induced by infection (Rainard *et al.,* 2022).

In view of the association of a wide variety of mastitogens and their antigenic diversity, many dairy researchers have deemed a polyvalent vaccine conceivably more pragmatic (Bradley *et al.,* 2015; Cunha *et al.,* 2020). To the end of the last century, a considerable degree of attempts has been made to investigate the role of polyvalent vaccines in the mastitis controlling strategies in dairy cows worldwide (Ruegg, 2017) but no reports on the development of such types of vaccines for dairy cows are available in Pakistan where the dairy industry is emerging and people are importing exotic cattle to establish corporate dairy farms. Although few reports on the evaluation of mastitis vaccines in buffaloes are available in Pakistan (Athar 2007; Guccione *et al.,* 2016) but no study is available for cows. Based upon the fact that in Pakistan, mastitis is caused by *S. aureus*, *Str. uberis* and *E. coli*, the development of an effective vaccine containing these three mastitogens and its mass use seem a national animal health imperative. Keeping in view the above scenario in Pakistan, this preliminary study had been designed to prepare and evaluate the primary, secondary and dose-dependent humoral immune response of inactivated polyvalent mastitis vaccines containing *S. aureus (tst), Strept. uberis (Cpn-60 targeted STUB)* and *E. coli (aggR)* in a rabbit model to be further replicated the said trials in our candidate animal i.e. dairy cow in order to control mastitis in dairy cows.

MATERIALS AND METHODS

Ethical approval: The research trials were conducted in compliance with the guidelines of the Ethical Review Committee of the University of Veterinary and Animal Sciences (UVAS) Lahore, Pakistan

Bacterial isolates: In this study, the most prevalent toxinotypes of *Staphylococcus aureus (tst)*, *Streptococcus uberis (Chaperonin-60 targeted STUB),* and *Escherichia coli (aggR)* were isolated from the milk of mastitic dairy cows. The bacterial isolates were procured from Animal Health Research Laboratory, Department of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore, Pakistan and further used for composite antigenic preparation. The procedures for isolation of *Staphylococcus aureus (tst), Streptococcus uberis (cpn-60 STUB),* and *Escherichia coli (aggR)* are in harmony with those of (Shome *et al.,* 2011; Raza *et al.,* 2015; Ariffin *et al*., 2019; Murrad *et al.,* 2020). The morphological and biochemical profile of these isolates were in agreement with previous studies (El-Jakee *et al*., 2008; Raza *et al*., 2015).

Determination of Immunogenicity of vaccinal isolates in rabbits: Firstly, immunogenic properties of selected field isolates of *S. aureus (tst), Strept. uberis (*Cpn-60 targeted STUB*)* and *E. coli* (aggR) were determined in rabbits while secondly a dose-dependent immune response was checked in rabbits. A total of 24 adult rabbits (New Zealand white strains, ~ 5 months of age and ~ 1.5 kg weight) were procured from the local market and were initially kept at Laboratory Animal House, Department of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore for 2 weeks for acclimatization purpose. The rabbits were fed a typical rabbit diet at 90gm/kg per day and offered clean drinking water *ad-libitum*. Then, the rabbits were divided randomly into 4 equal groups (A, B, C, and D) and were injected antigenic preparation.

Preparation of antigens: Vaccinal isolates of *S. aureus, Strept. uberis* and *E. coli* were grown separately in brain heart infusion broth at 37^{o}C for 24 hours. After checking the purity by gram's staining, the cultures were inactivated with formalin (0.4%, v/v) for 24 hours. The inactivated cells were harvested by centrifugation at 3,000 rpm for 30 minutes at 4^{o}C. The cells were washed with phosphate buffer saline (PBS; pH 7.2) twice and final sediments were resuspended in PBS. The concentration of each antigenic preparation was set at 10^{6} cells/mL using the spectrophotometric method (Hirch and Strauss, 1964) and McFarland standards.

Inoculation and evaluation of antigenic preparation in rabbits: The rabbits in group A were injected *S. aureus (tst)* antigen at the dose rate of 0.2 mL SC twice at weekly intervals. The rabbits in group B were given *Strept. uberis (Cpn-60 STUB)* antigen while members in group C were administered *E. coli (aggR)* antigen at the same dose rate. Serum samples from these inoculated rabbits were collected at weekly intervals following

completion of 2nd shot of the inocula of *S. aureus (tst), Strept. uberis (Cpn-60 STUB)* and *E. coli (aggR)* for 3 consecutive weeks (up to 28 days from the day of 1st injection). Antibody response to these antigenic preparations was evaluated by a commercially available iELISA kit (Abbexa, UK) at days 0 (pre-inoculation) and 7, 14, 21 28 (post-inoculation).

Evaluation of dose-dependent immune response to vaccinal isolates in rabbits: For the determination of the dose-dependent immune response of vaccinal isolates, another set of 18 adult rabbits was divided into 3 groups of 6 (A, B, and C). These rabbits were also kept for 2 weeks for acclimatization before the commencement of the experiment. In this experiment, composite antigens of different antigenic concentrations were prepared instead of separate antigens by the above-described procedure. The rabbits in group A were treated with composite antigenic preparation containing 10^8 cells/mL of each of the vaccinal isolates. To the rabbits in group B antigenic preparation containing 10^{10} cells/mL of each of the vaccinal isolates was administered. The rabbits in group C were given antigenic preparation having 10^{12} cells/mL of each of the vaccinal isolates. Serum samples from these rabbits were collected at weekly intervals following completion of 2nd shot of inocula of each dose for 3 consecutive weeks. The antibody response to each of the vaccinal isolates was evaluated using a commercially available iELISA kit (Abbexa, UK).

Statistical analysis: The data originating from different experimental trials 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 immunogenicity and dose dependent immune response of composite antigenic preparation in the treatment and control groups. For the comparison of significant treatment means, Duncan's multiple range test was applied.

RESULTS

In the current study immunogenicity of vaccinal isolates of *Staphylococcus aureus (tst), Streptococcus uberis (*Cpn-60 targeted STUB*), and Escherichia coli (aggR)* were determined through primary and secondary immune response measurements. The measurement was done separately through iELISA by using Abbexa Mastitis Kits. The Optical Density (O.D) values of iELISA antibody titer to the concentration of 10^6 cells/mL of each preparation containing *S. aureus (tst), Strept. uberis (Cpn-60 STUB) and E. coli (aggR)* respectively in twenty-four adult healthy rabbits divided randomly into 4 groups viz A, B, C and D having 6 rabbits in each group are shown in (Table 1). The vaccine isolates *viz Staphylococcus aureus (tst)*, *Streptococcus uberis* (*Cpn-60* STUB), *Escherichia coli (aggR)* and Placebo (Control) were inoculated subcutaneously at day 0 as priming dose and a booster dose at day 7 respectively. The serum antibodies titers were raised and recorded at days 0, 7, 14, 21, and 28 respectively by using commercially available iELISA Mastitis Kit (Abbexa, UK). In Group A, antibody titers against days 7, 14, 21, 28 showed the statistically significant difference ($p \leq 0.05$) with an increasing trend that peaks at day 14 (1.91 ± 0.02) then declination occurs showing significant difference at day 21 and day 28 against day-0, 7 of inoculation. For Group B and C, a similar trend with statistically significant different O.D values of serum samples ($p \leq 0.05$) was noticed against different sampling days. The antibody response was significantly higher in the post-booster samples at day-14 and day-21 for all the selected isolates. The highest antibody response (2.04 ± 0.13) was shown by Group B on day-14 (Table 1). All the groups viz A, B, C when compared with the control group (D) depicted the statistically significant difference ($p<0.05$).

Serum Indirect ELISA Antibody titers against composite antigenic preparations containing 3 different concentrations are given in (Tables 2, 3, 4). For all three vaccinal organisms, the rabbits of group B injected with 10^{10} cells/mL showed the highest antibody titer ($2.97^{A, a} \pm 0.10$) at day 14 of post-inoculation while selected vaccinal isolate in composite form were injected twice on day-0 and day-7. Serum antibody O.D Values (Mean±S.E) against *S. aureus (tst)* antigen in the rabbits of group B showed a significantly higher ($P<0.05$) difference as compared to those of groups A and C (Table 2).

Furthermore, for *Strept. uberis* (Cpn-60 targeted STUB) antigen, the titers of group B were higher ($p<0.05$) as compared to group A and C showing peak O.D value ($2.78^a \pm 0.07$) at day-14 (Table 3).

For *E. coli (aggR)* titers for groups, A and C were almost identical but both were statistically lower ($p<0.05$) than those of B ($2.84^a \pm 0.08$) (Table 4). This indicated that the immune response to these antigens in rabbits was dose-dependent for 10^8, 10^{10} and 10^{12} cells/mL.

Table 1. O.D Values (Mean±S.E) of Serum iELISA Antibody Titres against vaccinal isolates of *Staphylococcus aureus (tst)*, *Streptococcus uberis (*Cpn-60 targeted STUB*)* and *Escherichia coli (aggR)* injected twice at day-0 (priming) and day-7 (booster).

Group	O.D Values (Mean±S.E) at different Post-inoculation Days					p-value
	Day 0	Day 7	Day 14	Day 21	Day 28	
A (n=6)	$0.10^{d} \pm 0.06$	$1.53^{B,c} \pm 0.06$	$1.91^{A,a} \pm 0.02$	$1.67^{A,b} \pm 0.04$	$1.50^{A,c} \pm 0.05$	<0.0001
B (n=6)	$0.04^{c} \pm 0.00$	$1.74^{A,ab} \pm 0.14$	**$2.04^{A,a} \pm 0.13$**	$1.80^{A,ab} \pm 0.16$	$1.54^{A,b} \pm 0.19$	<0.0001
C (n=6)	$0.04^{d} \pm 0.00$	$1.71^{AB,b} \pm 0.08$	$1.98^{A,a} \pm 0.08$	$1.69^{A,b} \pm 0.12$	$1.34^{A,c} \pm 0.10$	<0.0001
D (n=6)	$0.04^{b} \pm 0.00$	$0.04^{C,a} \pm 0.00$	$0.04^{B,ab} \pm 0.00$	$0.04^{B,ab} \pm 0.00$	$0.04^{B,b} \pm 0.00$	0.0339
p-value	0.3980	<0.0001	<0.0001	<0.0001	<0.0001	

$^{A-C}$superscripts on different means within column differ significantly at $p \leq 0.05$
$^{a-d}$superscripts on different means within row differ significantly at $p \leq 0.05$
A= *S. aureus* (tst) antigen (10^6 cells/mL)
B= *Strept. uberis* (Cpn-60 targeted STUB) antigen (10^6 cells/mL)
C= *E. coli (aggR)*(10^6 cells/mL)
D= Control (Placebo)

Table 2. O.D Values (Mean±S.E) of Serum iELISA Antibody Titres against 3 different concentrations of vaccinal *Staphylococcus aureus (tst)* antigen to check dose dependent response in rabbits.

Group	O.D Values (Mean±S.E) against *S. aureus (tst)* antigen at different Post-inoculation Day					p-value
	Day 0 (1st shot of inoculum)	Day 7 (2nd shot of inoculum)	Day 14	Day 21	Day 28	
A (10^8 cells/mL) (n=6)	$0.042^{A,d} \pm 0.00$	$2.21^{B,b} \pm 0.06$	$2.52^{B,a} \pm 0.05$	$2.15^{B,b} \pm 0.07$	$1.84^{B,c} \pm 0.05$	<0.0001
B (10^{10} cells/mL) (n=6)	$0.041^{AB,d} \pm 0.00$	$2.66^{A,b} \pm 0.11$	**$2.97^{A,a} \pm 0.10$**	$2.68^{A,b} \pm 0.10$	$2.28^{A,c} \pm 0.07$	<0.0001
C (10^{12} cells/mL) (n=6)	$0.038^{B,d} \pm 0.00$	$2.45^{AB,b} \pm 0.07$	$2.71^{B,a} \pm 0.05$	$2.38^{B,b} \pm 0.07$	$2.15^{A,c} \pm 0.06$	<0.0001
p-value	0.0145	0.0061	0.0017	0.0009	0.0005	

$^{A-B}$superscripts on different means within column differ significantly at $p \leq 0.05$
$^{a-d}$superscripts on different means within row differ significantly at $p \leq 0.05$

Table 3. O.D Values (Mean±S.E) of Serum iELISA Antibody Titres against 3 different concentrations of vaccinal *Streptococcus uberis* (Cpn-60 targeted STUB) antigen to check dose dependent response in rabbits.

Group	O.D Values (Mean±S.E) against *Strept. uberis* (Cpn-60 targeted STUB) antigen at different Post-inoculation Day					p-value
	Day 0 (1st shot of inoculum)	Day 7 (2nd shot of inoculum)	Day 14	Day 21	Day 28	
A (10^8 cells/mL) (n=6)	$0.043^{A,d} \pm 0.00$	$2.36^{b} \pm 0.06$	$2.66^{a} \pm 0.06$	$2.26^{b} \pm 0.07$	$2.06^{c} \pm 0.04$	<0.0001
B (10^{10} cells/mL) (n=6)	$0.039^{B,d} \pm 0.00$	$2.54^{b} \pm 0.09$	**$2.78^{a} \pm 0.07$**	$2.56^{ab} \pm 0.10$	$2.25^{c} \pm 0.09$	<0.0001
C (10^{12} cells/mL) (n=6)	$0.044^{A,d} \pm 0.00$	$2.38^{b} \pm 0.12$	$2.70^{a} \pm 0.09$	$2.36^{b} \pm 0.06$	$2.09^{c} \pm 0.06$	<0.0001
p-value	0.0222	0.3407	0.4813	0.0562	0.1377	

$^{A-B}$superscripts on different means within column differ significantly at $p \leq 0.05$
$^{a-d}$superscripts on different means within row differ significantly at $p \leq 0.05$

Table 4. O.D Values (Mean±S.E) of Serum iELISA Antibody Titres against *3 different concentrations of vaccinal Escherichia coli (aggR)* antigen to check dose dependent response in rabbits.

Group	O.D Values (Mean±S.E) against *E. coli (aggR)* antigen at different Post-inoculation Day					p-value
	Day 0 (1st shot of inoculum)	Day 7 (2nd shot of inoculum)	Day 14	Day 21	Day 28	
A (10^8 cells/mL) (n=6)	$0.04^{d} \pm 0.00$	$2.46^{b} \pm 0.08$	$2.71^{a} \pm 0.06$	$2.47^{b} \pm 0.03$	$2.13^{c} \pm 0.06$	<0.0001
B (10^{10} cells/mL) (n=6)	$0.04^{d} \pm 0.00$	$2.60^{b} \pm 0.09$	**$2.84^{a} \pm 0.08$**	$2.53^{b} \pm 0.07$	$2.33^{c} \pm 0.07$	<0.0001
C (10^{12} cells/mL) (n=6)	$0.04^{c} \pm 0.00$	$2.58^{a} \pm 0.09$	$2.79^{a} \pm 0.08$	$2.52^{a} \pm 0.13$	$2.22^{b} \pm 0.12$	<0.0001
p-value	0.6872	0.4516	0.5168	0.8642	0.2885	

[a-d]superscripts on different means within row differ significantly at $p \leq 0.05$

DISCUSSION

Bovine mastitis has detrimental impact on dairy industry of Pakistan Vaccination can play a significant role in mastitis control programs. Vaccine development against common mastitogens has been advancing in the past few decades (Ismail, 2017). It promotes the formation of acquired immunity against mastitogens as well as has a low side effect (Zhylkaidar *et al.,* 2021). In view of the poly-microbial etiological nature of mastitis, polyvalent vaccine comprises the most common mastitis pathogens (*S. aureus, Strept. uberis* and *E. coli are* seen to have a wider application than monovalent vaccine (Ahmad and Ibrahim, 2016).

In order to evolve an effective vaccine to minimize the incidence of mastitis in the target species i.e. cattle, it is mandatory to evaluate the antigenic response against mastitogens in laboratory animals so that optimum antigenic dose of these organisms could be determined. Keeping in view the need of the hour, the present study was conducted to monitor the antigenic response of composite formalin inactivated *S. aureus (tst)*, *Strept. uberis (Cpn-60 STUB)* and *E. coli (aggR)* antigen preparation in rabbits. The ultimate objective of the study was to evaluate the experimental vaccine in cattle (the actual host of disease). Therefore, in this study, preliminary trials of immunogenicity and dose dependent immune response against said mastitogens were conducted in rabbits in order to proceed further for development of polyvalent mastitis vaccine.

The development of effective polyvalent mastitis vaccines is a dire need of time to combat antimicrobials' resistance. In this study, the primary immune response was noticed at day-7 of priming, while (Aqib *et al.,* 2018) reported the primary response against *S. aureus* at day 15. Consequently, the pathogenicity and antigenicity of field isolates were proved in terms of induced primary and secondary immune responses in rabbits. The results are in line with the findings of (Abubakar *et al*., 2006; Shakoor *et al*., 2006). But Arshed (2002) reported a peak in geomean antibody titer (GMT) during the 3rd and 4th week post-booster respectively, which then gradually declined up to day 60 post-booster. The indirect haem-agglutination antibody titers were raised significantly at the first week of booster in group B, which indicated the antigenic potential of the organism. Dad *et al.,* (2022) has described that the secondary immune response was more intense because the initial inoculation of antigen leads to the multiplication of responsive cells. This study investigated the increased antibody titer at booster dose which is in agreement with recommendations of OIE manual and findings of (Fattom *et al*., 2004; Shakoor *et al*., 2006; Farooq *et al.,* 2008; Aqib *et al*., 2018). Consequent to the findings of the present study it was concluded that *S. aureus (tst), Strept. uberis (*Cpn-60 targeted STUB*)* and *E. coli (aggR)* isolates showed antigenic response in rabbits. The antigenic response was higher in the animals getting a booster dose of *S. aureus (tst), Strept. uberis (*Cpn-60 targeted STUB*)* and *E. coli (aggR)* antigens as compared to the animals getting a single dose.

In the current study, the selected concentration (10^{10} Cells/mL) of *S. aureus* (tst) was found to be more immunogenic as compared to other concentrations. This is in agreement with the findings of (Aqib *et al.,* 2018). Antigenic concentrations of 10^8 Cells/mL and 10^{12} Cells/mL were experimentally proved to be less immunogenic than 10^{10} Cells/mL. These findings were in harmony with (Giraudo *et al.* 1997; Butt. 2006; Athar. 2007; Aqib *et al*., 2018a) in terms of selected concentration of 10^{10} Cells/mL for vaccinal isolated.

However, the current finding is contrary to the findings of (Aqib *et al*., 2018b), who reported that only

10^{12}Cells/ml was immuno-suppressive initially than the other two concentrations. Therefore, an increase in a concentration above 10^{10} cells/ml did not enhance the immune response, rather a decrease was observed. Several other workers (Ahmed and Ibrahim, 2016; Aqib *et al.,* 2018; Dad *et al.,* 2022) conducted dose-dependent trials of *S. aureus* in rabbits and concluded that a concentration above 10^{10} Cells/ml did not elicit a significantly higher immune response.

A study on primary and secondary antibody response (IHA antibodies) to *Streptococcus agalactiae* antigen by Abubakar *et al*., (2006) concluded that the double dose of *Streptococcus agalactiae* antigen in rabbits showed better and long-lasting humoral antibody response as compared to a single dose. The IHA antibodies (GMT) obtained their peaks at day 30 and day 45 post-inoculation (PI) with a gradual drop up to day 60 PI in groups A and B respectively. Aqib *et al*., (2018) has reported that the secondary immune response is more intense because the initial inoculation of antigen leads to the multiplication of responsive cells, which may persist for a long time in the animal. Similarly, Hambali *et al.* (2018) prepared *S. aureus* killed vaccine by culturing the bacteria in BHI broth and adding 0.5% formalin for complete inactivation, and prepared Aluminum Potassium Sulphate adjuvanted inactivated vaccine with four different antigenic concentrations i.e.10^6 CFU/mL, 10^7 CFU/mL, 10^8 CFU/mL, 10^9CFU/mL.

The present findings also supported the study of Raza *et al*. (2015) who stated that dose-dependent immune response can be elicited up to a definitive bacterial concentration depending upon the type of bacteria and nature of adjuvant used. The results of present study are in agreement with that of Avais *et al*., (2005) who concluded that the primary IHA antibody response to *E. coli* of composite Antigen was higher at day-15 compared with *S. aureus* and *S. agalactiae* whereas secondary antibody response to *S. aureus* was higher as compared with *Strept. agalactiae* and *E. coli* at day-45.

Conclusion: The *Staphylococcus aureus* (*tst*), *Streptococcus uberis* (*Chaperonin-60 targeted STUB*), and *Escherichia coli* (*aggR*) local field toxinotypes from cow mastitic milk were antigenic as well as more immunogenic. The concentration of 10^{10} cells/mL was found to be most efficacious due to sustained serum antibody titers for a specified period of time and hence selected for vaccine preparation. These promising results from all these three vaccinal concentrations warrant their trials in small and large ruminants for the control of mastitis.

Authors' Contribution: BAS*, MA**, JAK, MR, AAA, MAA conceived and designed the study. BAS performed experiments. MA and SHZ helped in sampling. BAS, MA and SA analyzed the data. SM helped in manuscript formatting. BAS wrote and critically reviewed the manuscript and all authors approved the manuscript.

Acknowledgements: Authors acknowledge PARB Funded Project No. 638 "Development of inactivated polyvalent vaccines for the control of mastitis in dairy cattle" for supporting this study.

Conflict of Interest: The authors declare no conflicts of interest regarding the publication of this article.

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