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Evaluation of humoral immunological response of newly prepared bovine multidrug-resistant *Staphylococcus aureus* mastitis vaccines in rabbits

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Abstract: Multi-drugs resistant (MDR) *Staphylococcus aureus* is creating challenges to cure cow mastitis, resulting in massive economic loss globally. It necessitates the adoption of prevention and control systems such as vaccination. Plain (PMRSAV), Montanide oil adjuvanted (MMRSAV) and Aluminum hydroxide adjuvanted (AMRSAV) vaccines were prepared using a molecularly characterized isolate of MDR *S. aureus* from bovine origin. Immunogenicity of the selected isolate was evaluated in five groups of rabbits (A-E) at different concentrations by measuring GMT via IHA from serum samples after booster shot. The group E provoked significantly higher ($P<0.05$) antibody titer with peak at day 28 (64±0.5) and cumulative mean antibody titer (CMT) of rabbits was highest (45.6) followed by groups C (35.9), D (32.7), B (30.3) and A (24.5). The concentration yielding maximum antibody titer was used for vaccines preparation. Vaccines were evaluated in different rabbits groups by inoculating PMRSAV, MMRSAV, AMRSAV and Placebo. Serum samples evaluated through IHA revealed that rabbits injected with MMRSAV produced highest antibody titer reaching its peak at day 45 (90.51±0.23) with a slight decrease until day 60 (80.63±0.17) followed by AMRSAV and PMRSAV. Challenge protection assay revealed the survival rates of rabbits in groups PMRSAV, MMRSAV, AMRSAV and Placebo as 83.3%, 100%, 100% and 16.7%, respectively. The study concluded that MMSAV and AMSAV were safe, efficacious and immunogenic in experimental rabbits.

Keywords: MDR *Staphylococcus aureus,* montanide oil adjuvanted vaccine, aluminum hydroxide adjuvanted vaccine, geometric mean titer, rabbits

INTRODUCTION

Mastitis is an inflammation of the mammary glands in dairy animals and remains the most economically important disease in the dairy industry in the developed and developing world (Wakchaure *et al.,* 2015; Ruegg, 2017; Aghamohammadi *et al.*, 2018). According to an article published by the University of Glasgow, mastitis is estimated to cost US$19.7 to US$32 billion annually to the global dairy industry. In United States, estimated losses to dairy industry due to mastitis are US$2 billion annually and it costs Canadian dairy industry more than US$310 million annually (University of Montreal study). Mastitis of *S. aureus* origin affects 50% of the dairy herd population in India, 47.5% in Pakistan, 51.3% of cows in Bangladesh, 54.3% in Zhejiang province of China and 52.5% of dairy cows in Egypt (Shaheen *et al.,* 2016).

The etiology of the disease is constantly changing, with the burden of new microbial species. About 150 bacterial species are isolated from mastitis cases in bovine (Shaheen *et al.,* 2016). Different types of bacteria, including *S. aureus, Str. agalactiae, E. coli, C. pyogenes, Str. dysgalactiae* and *Str. uberis*, are believed to be responsible for bovine mastitis (Radostits *et al.,* 2010). Retrospective analysis of epidemiological studies shows that *S. aureus* is responsible for approximately 50% of all mastitis cases in Pakistan (Athar, 2007).

The veterinary and agriculture components account for more than 50% of the global antibiotic consumption (Oliver *et al.,* 2011), reported as an important factor in the development of antimicrobial resistance (AMR) (McEwen and Fedorka-Cray, 2002; Li and Zhao, 2018). Antibiotics are used indiscriminately to treat bovine mastitis, and many antibiotics such as chloramphenicol, novobiocin, ciprofloxacin, oxytetracycline, gentamicin, oxacillin, penicillin, clindamycin, erythromycin and vancomycin are poorly effective against *S. aureus* (Saei, 2012; Deb *et al.,* 2013; Xu *et al.,* 2015; Wang *et al.,* 2016). The irrational and unnecessary use of antibiotics in veterinary medicine is the main factor behind the development of AMR and has become a public health issue (Tark *et al.,* 2017).

The emergence and spread of antibiotic resistance has been highlighted as a global threat by various health organizations and pathogens resistant to antibiotics are causing significant morbidity and mortality. With the emergence of resistance to multiple drugs, new and effective treatment and prevention approaches are needed (Micoli *et al.,* 2021). Vaccines can reduce the incidence and spread of AMR both directly and indirectly (Mishra

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et al., 2012; Klugman and Black, 2018). The use of vaccines reduces the incidence of the resistant pathogens and the use of antibiotics (Micoli *et al.,* 2021). In addition, decreased transmission of pathogens and infections in vaccinated entities significantly reduced antibiotic therapies and decreased the circulation of resistant strains (Lipsitch and Siber 2016). It has been reported that the use of vaccines in farm animals significantly reduces antibiotic consumption and reduces the risk of developing AMR (Hoelzer *et al.,* 2018). This could also have implications for human health as resistance determinants could be transferred to bacteria that infect humans or resistant pathogens could infect humans directly (Micoli *et al.,* 2021). This is the preliminary study concerning the development of a multidrug-resistant *S. aureus* vaccine against bovine mastitis, where we prepared and evaluated the humoral immune response and the protective effect of multidrug-resistant *S. aureus* mastitis vaccines rabbits.

MATERIALS AND METHODS

Source of Multidrug-Resistant Staphylococcus aureus (MDRSA)

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 antigen

MDRSA grown in brain heart infusion broth at 37°C for 24 hours was inactivated using formalin (0.4% v/v) for 24 hours. The inactivated cells were then harvested by centrifugation at 3000 rpm for 30 minutes at 4°C. The harvested cells were washed using phosphate buffer saline (PBS; pH 7.2) twice and final sediments were re-suspended in PBS. The concentrations of antigenic preparations were set at 10^6, 10^7, 10^8, 10^9 and 10^{10} cells/mL using spectrophotometric method (Hirsch and Strauss 1964).

Determination of immunogenicity of MDRSA in rabbits

To determine immunogenicity of MDRSA, 20 adult male locally bred rabbits were divided into 5 groups of 4 viz. A-E. Rabbits in group A were given MDRSA antigenic preparation containing 10^6cells/mL intraperitoneally (i.p.) while group B, C, D and E were administered 10^7, 10^8, 10^9 and 10^{10} cells/ml, respectively. A booster dose of respective antigenic preparation was administered 2 weeks apart.

Serum samples were collected on weekly basis for 6 consecutive weeks following booster shot. The antibody titer against MDRSA was determined through Haemagglutination inhibition (IHA) assay (Rahman *et al.,* 2005). Antigenic concentration with highest antibody titer was used in vaccine preparation.

Preparation of plain and adjuvanted MDRSA mastitis vaccines

The vaccines were prepared according to the procedures and protocol as described by (Dad *et al.*, 2022).

Evaluation of MDRSA mastitis vaccines in rabbits

For monitoring safety and side effects of MDRSA mastitis vaccines, 24 adult locally bred rabbits procured from local market were divided into 4 groups of 6. Half of the rabbits in each group were injected 0.2 mL of vaccine SC while other half of rabbits were inoculated 0.5 mL of vaccine SC. Any local or systemic reaction was recorded for up to 7 days.

For evaluation of humoral response to MDRSA mastitis vaccines, 24 adult healthy male rabbits were divided into 4 groups (A-D) with 6 rabbits in each. Rabbits in group A were vaccinated plain MDRSA mastitic vaccine at 0.2 mL SC and rabbits in group B were given Montanide adjuvanted MDRSA mastitis vaccine 0.2 mL IM. The rabbits in group C were injected aluminum hydroxide adjuvanted MDRSA mastitis vaccine at 0.2mL SC. The rabbits in group D were administered placebo at 0.2mL SC and served unvaccinated control. A booster dose was injected to rabbits in each group after 15 days. Serum samples were collected from rabbits in each group fortnightly for up to 6 weeks and serum antibody titers were measured against MDRSA using IHA test as described by Rahman *et al.* (2005).

For challenge protection assay another set of 24 adult healthy rabbits were divided into 4 groups of 6 (A-D). Rabbits in four groups were vaccinated twice with 0.2mL dose of each of MDRSA mastitis vaccine at 15 days interval while rabbits in group D were given placebo only. At day 30 of post-booster vaccination, rabbits in each group were challenged with 1mL of live inoculum ($1x10^{10}$cells/mL) of MDRSA. Any morbidity and mortality were recorded till day 7 post challenge and percent survival rate was calculated (Athar, 2007).

Ethical approval

The experimentation on use of animals was approved by Ethical Review Committee of University of Veterinary and Animal Sciences, Lahore, Pakistan. It is further confirmed that these experiments were conducted according to established animal welfare guidelines and study complies with all regulations.

STATISTICAL ANALYSIS

Vaccinated and control groups were compared by computing geometric mean titers and also by cumulative mean titers.

RESULTS

Dose-dependent immunogenic response

All the antigen concentrations provoked considerable immune response starting from day 7 with a peak at day 28 and then gradual decrement till day 42. Statistical analysis revealed significant difference ($P<0.05$) among GMT values at different days. Rabbits in group E exhibited significantly higher ($P<0.05$) antibody titers at various days with peak at day 28 (64±0.5) compared to other rabbits in other groups. Likewise, cumulative mean antibody titers (CMT) of rabbits in this group was highest (45.6) followed in order by rabbits in groups C (35.9), D (32.7), B (30.3) and A (24.5). These results clearly demonstrated that selected MDRSA isolate was immunogenic and produced dose dependent immune response in rabbits with highest antibody titers revealed by antigenic concentration of 10^{10}cells/mL (fig. 1 & 2). Hence, 10^{10}cells/mL antigenic concentration was used in vaccine preparation.

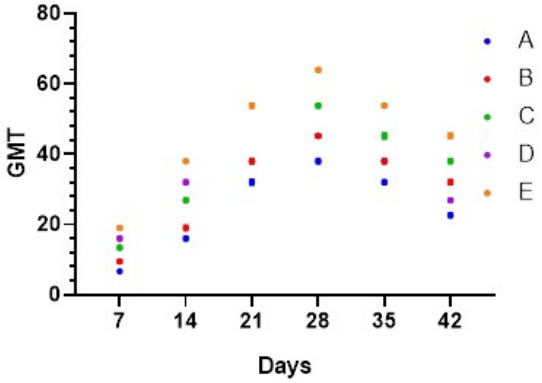

Fig. 1: Humoral immune response to various antigenic concentrations of multidrug resistant *S. aureus* at different days

Sterility and side effect of vaccines

Three types of vaccines namely plain multidrug resistant *S. aureus* vaccine (PMRSAV), Montanide adjuvanted multidrug resistant *S. aureus* vaccine (MMRSAV) and aluminum hydroxide adjuvanted multidrug resistant *S. aureus* vaccine (AMRSAV) were prepared and streaked on blood agar plates to test for sterility and contamination. After incubation of 48 hrs at 37°C, absence of any growth on blood agar revealed that vaccines were sterile and free from contamination. Results of safety of vaccines in rabbits are shown in (table 1). Mild to moderate injection site swelling was developed in one rabbit in response to subcutaneous administration of higher (0.5ml) dose of PMRSAV. Similarly, moderate to severe injection site swelling was observed in one rabbit of group C when administered 0.5 mL dose of AMRSAV, subcutaneously. However, these swellings in both the rabbits subsided within 48 hours. Safety data demonstrated that the vaccines did not provoke any significant local or systemic reaction in rabbits, hence, were considered safe.

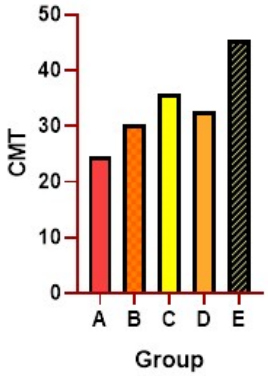

Fig. 2: Cumulative mean antibody titers of various antigenic concentration of multidrug resistant *S. aureus* in rabbits

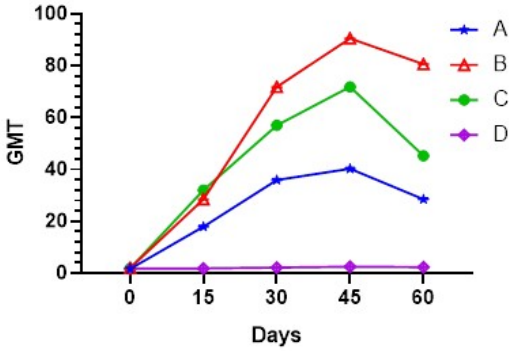

Fig. 3: Humoral immune response to bovine multidrug resistant *S. aureus* mastitis vaccines in rabbits at different days

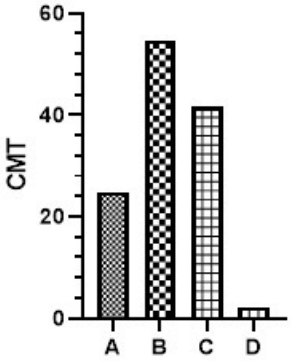

Fig. 4: Cumulative mean antibody titer in bovine multidrug resistant *S. aureus* vaccinated and control group rabbits

Humoral immune response of vaccines

Geometric mean antibody titer (GMT) of rabbits in group B (MMRSAV) began to rise from day 15 (28.51±0.27) and reached its peak at day 45 (90.51±0.23) with a slight decrease until day 60 (80.63±0.17) followed by the rabbits in group C (AMRSAV) that produced higher antibody titer at day 15 (32±0.22) with peak at day 45 (71.84±0.31) and then a sharp decline was observed at day 60 (45.25±0.19). On the other hand, antibody titers were the lowest in rabbits in group A (PMRSAV) that peaked at day 45 (40.31±0.17) followed by sharp decline

Table 1: Safety data of rabbits in vaccinated and control groups

Group	Vaccine type	No. of Rabbits	Dose and Route		Mortality	Morbidity	Symptoms
A	PMRSAV	6	3	0.2ml SC	0	0	-
			3	0.5ml SC	0	1	Mild/Moderate
B	MMRSAV	6	3	0.2ml SC	0	0	-
			3	0.5ml SC	0	0	-
C	AMRSAV	6	3	0.2ml SC	0	0	-
			3	0.5ml SC	0	1	Moderate/Severe (+)
D	Placebo	6	3	0.2ml SC	0	0	-
			3	0.5ml SC	0	0	-

Table 2: Survival rate of rabbits after challenge with live inoculum of MDRSA in vaccinated and control groups

Group	Vaccine	No. of Rabbits		Survival rate (%)
		Total Rabbits	No. of dead rabbits within 7 days	
A	PMRSAV	6	1	83.3
B	MMRSAV	6	0	100
C	AMRSAV	6	0	100
D	Placebo	6	5	16.7

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

at day 60 (28.51±0.11). Statistical analysis revealed significant difference ($P<0.05$) in GMT of vaccinated and control group rabbits at various days. When compared GMT of rabbits in various groups, significantly higher ($P<0.05$) antibody titers were observed in rabbits of groups B (MMRSAV) followed in order by group C (AMRSAV) and A (PMRSAV) (Fig. 3). Likewise, cumulative mean antibody titers (CMT) of rabbits in group B (MMRSAV) were higher (54.7) compared to rabbits in groups C (41.6) and A (24.9) (Fig. 4).

Challenge protection assay revealed survival rates of 83.3%, 100%, 100% and 16.7% of rabbits in groups A, B, C and D, respectively (Table 2).

DISCUSSION

Staphylococcus aureus represents one of the leading causes of mastitis in dairy cows worldwide. *S. aureus* IMI have variable outcomes due to virulence of the strain involved, immune defenses of the host, and by antibiotic resistance (Scali *et al.,* 2015). The detection of MDRSA in mastitis milk is alarming and represents a great public health concern (Salauddin *et al.,* 2020). Multiple approaches are needed to prevent infections and reduce the use of antimicrobial drugs. Among these, vaccines are effective tools to prevent infections and they have the potential to make a major contribution to the control and prevention of AMR (Scali *et al.,* 2015).

Humoral immune response against several concentrations of MDRSA isolate was reported to be dose-dependent in rabbits and the highest immune repose was provoked at concentration 10^{10} cells/mL. A dose-depnedent humoral immune response to *S. aureus* was reported by other workers with hgihest antibody titers at bacterial concentration of 10^{10} cells/mL (Watson *et al.,* 1996; Opdebeeck and Norcross 1985; Shakoor 2006; Athar 2007). Moreover, the humoral immune response to MDRSA was increased when booster dose was given to rabbits which corresponds to the findings of (Haba and Nisonoff 1985; Fattom *et al.,* 2004; Avais *et al.,* 2007; Aqib *et al.,* 2018). Hence, bacterial concentration of 10^{10} cells/mL was used for vaccine preparation which is in agreement with other workers (Shakoor *et al.,* 2006; Aqib *et al.,* 2018). Giraudo *et al.,* (1997) and Butt (2006) demonstrated that bacterial concentartions above 10^{10} cells/mL are immunosuppressive.

All the vaccines prepared in this study were sterile and free from contamination. These vaccines did not provoke any adverse effect in rabbits and were safe to use which is congruent with the findings of other workers (Yousaf *et al.,* 2009; Aqib *et al.,* 2018). However, Plain and aluminum hydroxide adjuvanted vaccines at high dose (0.5mL) resulted into slight injection site swelling that subsided within 48 hours. Similar results were observed by (Butt 2006; Ahmad and Muhammad 2008).

In the present study, rabbits vaccinated with Montanide adjuvanted MDRSA vaccine provoked higher humoral immune response (GMT) compared to aluminum hydroxide adjuvanted MDRSA vaccine or plain MDRSA vaccine. Statistical analysis revealed significant difference ($P<0.05$) in humoral immune response of vaccinated and control group rabbits. Similarly, (Aqib *et al.,* 2018) demonstrated that rabbits vaccinated with Montanide adjuvanted MDRSA vaccine produce high antibody titers compared to Alum precipitated MDRSA vaccine.

Our study demonstrated highest humoral immune response at day 45 and then steady decline till day 60 which is in agreement with the findings of other workers (Nordhaug *et al.,* 1994a; Han and Park 2000). The persistent antibody titer in case of Montanide adjuvanted MDRSA vaccine is the result of depot formation and then slow release of antigen (Nordhaug *et al.,* 1994b). Furthermore, it was observed that humoral immune response in rabbits vaccinated with Montanide MDRSA vaccine was more persistent till the end of the experiment compared with aluminum adjuvanted or plain MDRSA vaccinated groups where a sharp decline in humoral immune response was observed after day 45.

Similarly, Aqib *et al.,* (2018) reported higher and persistent immune response to Montanide adjuvanted vaccines than alum precipitated ones. Similarly, higher IHA antibody titers were observed in animals injected with live attenuated, dextran sulphate adjuvanted and oil-adjuvanted *S. aureus* vaccines than plain (Shakoor *et al.,* 2006). Challenge protection assay in rabbits revealed that vaccinated rabbits had higher survival rate compared to non-vaccinated rabbits and these findings are consistent with the results of other workers (Giraudo *et al.,* 1997; Butt 2006; Ahmad and Muhammad 2008; Aqib *et al.,* 2018).

CONCLUSION

Numerous health organizations have highlighted the global concern about the emergence and spread of antibiotic resistance, which is one of the leading causes of morbidity and mortality from antibiotic-resistant bacteria. The emergence of multidrug resistance requires the development of new and efficient preventive and treatment strategies. Vaccines can both directly and indirectly stop the emergence and spread of AMR. Multidrug-resistant bovine *S. aureus* elicited a dose-dependent humoral immune response in rabbits and the MDRSA-prepared vaccines were sterile and safe for use in animals. In addition, Montanide-adjuvanted MDRSA vaccine was more effective in terms of increasing persistent humoral immune response and survival rate in rabbits compared to aluminum hydroxide-adjuvanted or plain MDRSA vaccines. The use of MDRSA bovine mastitis vaccines can reduce severity, new IMI and prevent antimicrobial resistance. The decreased transmission of pathogens and infections in the vaccinated entities can significantly reduce antibiotic therapies and decrease the circulation of resistant strains.

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