Evaluation of humoral immunity and protective efficacy of biofilm producing *Staphylococcus aureus* bacterin-toxoid prepared from a bovine mastitis isolate in rabbit

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Summary

Mastitis is a one of the major diseases of dairy animals. *Staphylococcus aureus* is the most common microorganism associated with this dairy scourge. Cure rates of mastitis associated with this pathogen are appallingly low. Biofilm is an important virulence factor and immunogenic structure of *S. aureus* that makes it resistant to phagocytosis and antibiotics. Reports on the efficacy of vaccine prepared from a biofilm producing *S. aureus* are infrequent. The present study was designed to evaluate the role of a bacterin-toxoid prepared from a strong biofilm producing *S. aureus* in effective immunization of rabbits. The strong biofilm producing *S. aureus* selected from 64 isolates of staphylococci was used to prepare bacterin-toxoid and aluminum hydroxide gel was added as an adjuvant. The vaccine was evaluated in rabbits by challenge protection assay and humoral immune response. The mortality rates in control and vaccinated groups were 80% and 10% at day 7 post challenge and 100% and 20% at day 15 post challenge, respectively. Serum antibody titer (GMT) was significantly higher (294.0) in vaccinated group as compared to control group of rabbits (2.63) at day 45. The results showed that the vaccine has significantly elicited humoral immune response in rabbit and developed protective efficacy against new infections.

Key words: Mastitis, *Staphylococcus aureus*, Biofilm production, Immune response, Rabbit

Introduction

Bovine mastitis is one of the most common infectious diseases of dairy animals that affects both quality and quantity of milk (Perez *et al*., 2009; Raza *et al*., 2013). Following the findings of field studies of economically substantial livestock diseases, mastitis is among the most important health problems of dairy animals (Hussain *et al*., 2005). Although mastitis can be caused by 137 microorganisms (Fux *et al*., 2005; Ranjan *et al*., 2006), *Staphylococcus aureus* is considered to be the number one mastitis pathogen, other microorganisms which may be responsible for mastitis include *Streptococcus agalactiae*, *Streptococcus uberis*, *Enterobacter aerogenes*, *Actinomyces pyogenes*, *Escherichia coli*, *Klebsiella* spp., some mold and yeasts (Gruet *et al*., 2001).

Biofilm is a structural community of bacterial population in which they are enclosed and composed of self-produced polymeric matrix (Prakash *et al*., 2003; Fux *et al*., 2005). Biofilm production by *S. aureus* is an important virulence and immunogenic factor. Studies showed that biofilm producing bacteria exhibited 10-1000 times resistance to antibiotics as compared to their counterpart planktonic bacteria (Olson *et al*., 2002; Melchior *et al*., 2007; Dhanawade *et al*., 2010). Isolates of *S. aureus* resistant to antibiotics and phagocytosis lead

to failure of the treatment so the development of vaccines against mastitis to protect from new infections by *S. aureus* is of valuable interest to the commercial milk producers. Vaccines used against *S. aureus* give variable results depending upon nature of vaccine, adjuvants used and some other factors (Watson and Davies, 1993).

An extensive variety of mastitis vaccines including inactivated bacteria with toxoid (Opdebeeck and Norcross, 1984), bivalent (*S. aureus* and *S. agalactiae*) bacterin-toxoid with aluminium hydroxide as adjuvant (Ahmad and Muhammad, 2008), bacteria encased in a mucous substance called a pseudo-capsule (Watson and Davies, 1993), capsular polysaccharide (CP) types CP5, CP8 and CP336 linked to protein carriers (von Eiff *et al*., 2007) and a concoction of slime in liposomes, toxoid and different inactivated bacteria (Amorena *et al*., 1994) have been investigated

Vaccines have revealed a considerable degree of protection against *S. aureus* mastitis. Recently, it was reported that bacterins from strong biofilm producing bacteria triggered the highest production of antibodies against Poly-N-acetylglucosamine (PNAG) and conferred the highest protection against mastitis in sheep compared to weak biofilm producing strain (Perez *et al*., 2009). It has been opined that a multicomponent vaccine incorporating a number of surface proteins and surface polysaccharides would prove to be more effective to

control mastitis in dairy animals (Schaffer and Lee, 2008).

There are limited reports available involving the role of biofilm in successful stimulation of protective immune response against *S. aureus* throughout the world. Based on these observations, the present study postulated that a mastitis vaccine prepared from a local strain of strong biofilm producing isolate of *S. aureus* could be effective, so the study was designed to evaluate the vaccine in rabbit model.

Materials and Methods

Isolation and identification of *Staphylococcus aureus*

Surf Field Mastitis Test (Muhammad *et al*., 2010) positive milk samples were collected aseptically as per the guidelines of National Mastitis Council (NMC, 1990) after screening of study population (n=192 animals) in addition to a set of 14 milk samples submitted to the laboratory.

The fresh samples were cultured and *Staphylococcus* isolates were presumptively identified following the standard guidelines (NMC, 1990). The staphylococcal isolates positive for tube coagulase test, protein A, clumping factor and certain exo-polysaccharides were further bio-typed by using a commercial identification kit (api® Staph). A 7 digit numeric profile (6716153) was generated using api® STAPH Identification Codebook by transforming the biochemical reactions on api® Staph kit into the numeral digits.

Detection of biofilm production by *Staphylococcus aureus* isolates

All the isolates were subjected to tube method (TM) (Christensen *et al*., 1982) and micro-plate (MP) assay (Mathur *et al*., 2006) for qualitative and quantitative detection of biofilm production, respectively. Based on these two tests, 6 isolates were short listed to perform Congo red agar (CRA) method (Mathur *et al*., 2006) to further confirm the trait. The vaccine isolate *S. aureus* (C.B-732 RR) was selected on the basis of TM, MP and CRA (data not shown) and hemolytic pattern of the isolates (Table 1).

Preparation of aluminum hydroxide adjuvanted bacterin-toxoid

Strong biofilm producing isolate of *S. aureus* was selected as the candidate vaccine isolate. The vaccine was prepared by adopting the protocol as described earlier (Giraudo *et al*., 1997; Ahmad and Muhammad, 2008). In order to provide the optimum cultural conditions, selected isolate of *S. aureus* was grown onto blood agar plates and then inoculated in modified nutrient broth (nutrient broth containing 10% w/v bubaline whey) for maximum encapsulation of *S. aureus*. The bacterial mass culture was inactivated with formalin (0.4% v/v) and the inactivated bacterial growth was centrifuged at 6,000 rpm for 30 min at 4°C. The supernatant was collected, autoclaved (121°C, 15 min) and stored at 4°C for further use as toxoid. The pellets of bacterial growth were re-suspended in phosphate buffer saline. The crude toxin extract (toxoid) and the preservatives (sodium azide 0.001% w/v, thimerosal sodium 0.001% w/v and formalin 0.4% v/v were added to the vaccine. Finally, aluminum hydroxide gel (Oxoid®) was added to the vaccine as an adjuvant and then final bacterial concentration was adjusted to 1×10^9 cells/mL of vaccine using spectrophotometer. The vaccine was checked for sterility and safety by inoculating vaccine onto the different growth media and administration of vaccine to rabbits and dairy cow (Giraudo *et al*., 1997).

Evaluation of bacterin-toxoid in rabbits

Twenty five adult healthy rabbits were divided into 2 groups (R1: control group included 10 rabbits and R2: vaccinated group included 15 rabbits). The rabbits in R1 and R2 were administered with 2 shots of placebo and vaccine at a dose of 0.2 mL at thigh region through intra-muscular route 15 days apart, respectively (Ahmad and Muhammad, 2008). The serum samples were collected from rabbits after every fortnight for a period of 2 months post-vaccination (0, 15, 30, 45 and 60 days) and inactivated by heating in water bath at 56°C for 30 min (Rahman *et al*., 2005).

At day 30 post second shot of vaccine, all rabbits of R1 (R1-1 thru R1-10) and 10 rabbits out of 15 of R2 (R2-1 thru R2-10) were challenged by injecting the

Table 1: Selection criteria for a vaccine isolate

Isolate I.D.	TM score[*]	MP score[**]	CRA results[**]	Hemolysis
Cow-708 RR	2	0.275	Weak	β
C.B-791 LF	3	0.227	Weak	β
C.B-732 RR	3	0.298	Moderate	β
Cow-138 LF[+]	3	0.015	Strong	β
Cow-474 RF[++]	3	0.223	Moderate	Non-hemolytic
Budhi Wachi RR[+++]	0	0.132	Strong	β

[*] Christensen *et al*. (1982) (basis for TM score: 0 = absent; 1 = weak; 2 = moderate; 3 = strong biofilm production), and [**] Mathur *et al*. (2006); Basis for MP score: OD (570 nm) values measured by spectrophotometer and if OD>0.10 = None, OD 0.11-0.15 = weak, OD 0.16-0.25 = moderate and OD<0.26 = strong biofilm production. [+] Isolate was dropped from selection as in MP assay it was negative for biofilm production, [++] Isolate was strong biofilm producer in all tests but was non-hemolytic, and [+++] Isolate was strong biofilm producer in CRA but negative in TM. Whereas LF means left front teat, LR means left rear teat, RF means right front teat and RR means right rear teat

active inoculum of *S. aureus* (1×10^9 cfu mL^{-1}) at a dose of 0.2 mL through intra-peritoneal route. Five rabbits of R2 (R2-11 thru R2-15) were not challenged as they were used for serum collection at day 60 after second shot of vaccine. Both groups were monitored for mortality up to 15 days post challenge. Indirect Hemagglutination (IHA) Test was performed for serological monitoring of antibodies against the bacterin-toxoid mastitis vaccine (Rahman *et al.*, 2005).

The research was conducted considering all the national and institutional legislations regarding animal protection and welfare. The use of the rabbits in the present experimental study was approved by the Directorates of Research and Advanced Studies and Society of Ethics of Animals, University of Agriculture, Faisalabad.

Data analysis

Geometric mean titers (GMT) were computed for both vaccinated and non-vaccinated control groups and then compared. Furthermore, cumulative mean titers and percentage values for challenge protection assay were calculated (Thrusfield, 2007).

Results

Prevalence of mastitis and different pathogens

The overall prevalence of clinical and sub-clinical mastitis in the study population was 48.44% (93/192 animals). A total of 107 isolates of 6 different genera were recovered on microbiological examination (Table 2).

Safety and sterility of the vaccine

The absence of any obvious growth on blood agar and MacConkey's agar plates up to 48 h indicated that vaccine was sterile and free from any contamination. There was no local or systemic reaction in rabbits and cow.

Evaluation of vaccine in rabbits
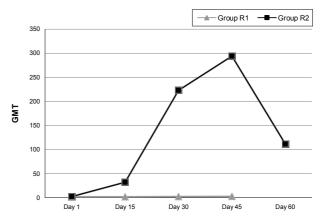*Challenge with live inoculums of vaccine isolate*

The vaccine showed a considerable survival rate in vaccinated group of rabbits compared to that of control group. The mortality rates and survival percentages in groups R1 and R2 are summarized in Table 3.

Humoral immune response

The GMT values of both groups are shown in Fig. 1. The antibody titer was highest (294.0) at day 45 which decreased gradually (111.4) at day 60 (Fig. 1). Cumulative mean titer was significantly higher in vaccinated group of rabbits (132.48) as compared to that of control group (2.20).

Discussion

It is now fairly well recognized that mastitis is one of the most expensive diseases of dairy animals all over the

Fig. 1: Comparison of humoral immune response in vaccinated and control (placebo control) groups of rabbits at different time points. Each data point shows the geometric mean titer (n=3)

Table 2: Frequency distribution of micro-organisms (n=107 isolates)

Pathogen/pathogen group	No. of isolates	Percentage (%)
Staphylococcus aureus (coagulase positive at 4 h)	43	40.18
Staphylococci (coagulase negative)	21	19.62
Corynebacterium species	15	14.01
Yeast	13	12.14
Bacillus species	7	6.54
Streptococcal species	6	5.60
Coliforms	2	1.86

Table 3: Results of challenge-protection assay at day 7 and 15 post vaccination

Groups of rabbits		R1	R2
Vaccination dose and route		Placebo 0.2 ml I/M[*]	Bacterin-toxoid 0.2 ml I/M[*]
Challenge dose (0.2 mL) and route of inoculation		Live inoculum (1×10^9 cells mL^{-1}) I/P[**]	Live inoculum (1×10^9 cells mL^{-1}) I/P[**]
No. of rabbits	Total rabbits	10	10
	Rabbits died in 7 days	8	1
	Rabbits died in 15 days	10	2
Protection or survival (%) till day 7 post challenge		20	90
Protection or survival (%) till day 15 post challenge		0	80
Mortality (%) after 7 days		80	10
Mortality (%) after 15 days		100	20

[*] Intra-muscular, and [**] Intra-peritoneal

world (Hortet and Seegers, 1998). *Staphylococcus aureus* has been reported as the most important mastitis pathogen in various countries across the world (Ali *et al.*, 2008; Kheirabadi *et al.*, 2008; Sindhu *et al.*, 2010). The results of present study indicated that *S. aureus* was the most prevalent microorganism in sub-clinical and clinical mastitis and the results are harmonious with previous studies (Gianneechini *et al.*, 2002; Ericsson *et al.*, 2009). Morphological and biochemical profile of the isolates coincided with previous reports (El-Jakee *et al.*, 2008).

As *S. aureus* infections respond poorly to antibiotics, vaccines are considered as solid cavalry against this microorganism and focus is now on enhancing the production of specific antibodies against PNAG and pseudo-capsular antigens (Watson, 1992; Nordhaug *et al.*, 1994). It has been reported that vaccines containing PNAG give considerable protective immune response and trigger antibodies production against *S. aureus* (Perez *et al.*, 2009). The selected vaccine isolate was grown in modified nutrient broth to provide the optimum cultural conditions as reported in previous studies (Watson and Watson, 1989). The capsule and/or extra-cellular glycocalyx is a polysaccharide which increases virulence of organism by impairing complement system and inhibiting antibody mediated opsonization (Fournier, 1990). Glycocalyx expression is usually ceased when the bacteria is allowed to grow in ordinary growth media, however, addition of bubaline whey to nutrient broth enhances the expression of glycocalyx (Dalhof, 1985; Watson and Watson, 1989) which is a component of bacterial biofilms. Aluminum hydroxide was used as an adjuvant whereas it is well established that aluminum hydroxide is capable of eliciting protective immune response (Perez *et al.*, 2009). All the procedures and protocols adopted for the development of bacterin-toxoid mastitis vaccine were in line with Giraudo *et al.* (1997) and Ahmad and Muhammad (2008).

Dose dependent immune response can be elicited up to a definitive bacterial concentration depending upon type of bacteria and nature of adjuvant used (Opdebeeck and Norcross, 1985). Although vaccines for mastitis against *S. aureus* have been extensively studied, there are substantial improvements in production of different vaccines. In past times, the major aim of using vaccines was to decrease the intensity of disease caused by *S. aureus* as most vaccines were not protective against new infections (Chang *et al.*, 2008; Middleton, 2008). Presently, with the advancement of new expertise, the major objective is to obtain greater degree of protection from novel infections. Some efforts have been made to develop vaccines containing purified PNAG capable of inducing some degree of protection against *S. aureus* new infections in sheep while it failed to stop the establishment of new infections in cows (Perez *et al.*, 2009; Prenafeta *et al.*, 2010). The challenge protection assay showed a considerable protective immune response in rabbits. The findings were in agreement with Giraudo *et al.* (1997) and Ahmad and Muhammad (2008) with significantly higher survival percentage in vaccinated

rabbits compared to that of rabbits in control group. The fallouts of challenge protection assay suggested that vaccine is capable of eliciting protective immune response and prevent further new infections of *S. aureus*.

Recently, it has been shown that vaccines containing PNAG induce strong antibody production in mouse model and cows (Perez *et al.*, 2009; Prenafeta *et al.*, 2010). Previous studies showed that bacterin-toxoid prevent development of new *S. aureus* infection in cows probably due to increased opsonization, increased phagocytic activity of polymorphonuclear cells as a result of augmented specific IgG antibodies against *S. aureus* in serum and milk (Watson, 1976; Pellegrino *et al.*, 2010). The chronological production of antibodies against biofilm producer *S. aureus* was increasing from day 1 up to day 45 and then followed by a plummet at day 60. Recently, it was shown that vaccine prepared from biofilm producing *S. aureus* produce significantly higher IgG level in rabbits as compared to that of free cell *S. aureus* vaccines and the study showed clear superiority of biofilm cells vaccine over free cells vaccine (Rathnamma, 2013). Similar type of antibody production response has been reported using biofilm producing *E. coli* vaccine in rabbits (Jyothi, 2013). The upshots of the study were in agreement with that of Han and Park (2000) who also observed a similar type of immune response against *S. aureus* in rabbits. The highest immune response was observed at days 30 and 45 post vaccination whereas some previous studies showed the highest immune response at days 45 and 60 (Han and Park, 2000; Ahmad and Muhammad, 2008) in rabbits. The vaccine prepared from strong biofilm producing isolate of *S. aureus* showed more effective results in challenge protection assay and a four-fold increase in serum antibody titer (GMT) when compared to the outcomes of a previous study (Ahmad and Muhammad, 2008) in which our laboratory workers used isolate of *S. aureus* (api[®] STAPH numeric profile 6736153) that showed weak biofilm production when evaluated for biofilm production in the present study (data will be published in separate publication).

The results of the study showed an increased antibody production in vaccinated group that was capable of preventing establishment of new *S. aureus* infection in rabbits as compared control group. Based on the results of present study, a short term clinical trial was conducted in dairy cows and buffaloes which also showed effectiveness of vaccine indicated by significant difference in prevalence and incidence of mastitis, high level of variation in microbiological examination of milk, reduced intra-mammary infections and somatic cell counts between vaccinated and control group of dairy cows and buffaloes (data will be shown in separate publication).

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