Pak. J. Agri. Sci., Vol. 45(2), 2008

EVALUATION OF *Staphylococcus aureus* AND *Streptococcus agalactiae* ALUMINIUM HYDROXIDE ADJUVANTED MASTITIS VACCINE IN RABBITS

Tanveer Ahmad and Ghulam Muhammad
Department of Clinical Medicine and Surgery, University of Agriculture Faisalabad

The aim of present study was to prepare and evaluate *Staphylococcus aureus* and *Streptococcus agalactiae* aluminium hydroxide adjuvanted mastitis vaccine in rabbits. For this purpose, isolates of these two bacterial species were recovered from aseptically collected milk samples (n=95) of mastitic buffaloes, identified and bio-characterized. Pathogenicity, immunogenicity, and antibiotics sensitivity testing of vaccinal organisms was performed. A bivalent aluminium hydroxide adjuvanted mastitis vaccine was prepared in Mastitis Research Laboratory, Department of Clinical Medicine and Surgery. The vaccine was found stable, sterile and safe to use. Quality of vaccine and its antibody response was evaluated in rabbits. Rabbits were divided into two groups (G_A and G_B) of ten rabbits each. Rabbits in group G_A were injected with *S. aureus* and *Str. agalactiae* aluminium hydroxide adjuvanted mastitis vaccine while rabbits of group G_B served as non-vaccinated control. Vaccine was found stable, sterile and safe to use. Indirect haemagglutination inhibition test was performed for determination of titers against vaccinal *S. aureus* and *Str. agalactiae*. Serum antibody titres (GMT) against S .*aureus* were highest (78.8) at day 45 in the rabbits of group G_A which declined slightly to 73.3 on day 60. The gradual increase in IHA titre for *Str. agalactiae* was also observed at day 45 and day 60 post vaccination. Cumulative mean antibody titre (CMT) was 44.94 for vaccinal *S. aureus* isolate and 46.56 for vaccinal *Str. agalactiae* isolate while in control group (G_B) 2.12 CMT was recorded. The antibody response of vaccine in terms of geometric mean titres was significantly higher in vaccinated group at day 45 and 60 post vaccination as compared to control group.The results of the study indicated that the bivalent(*S. aureus* and *Str. Agalactiae*) aluminium hydroxide adjuvanted vaccine was immunogenic in rabbits.

Keywords: Mastitis vaccine, *Staphylococcus aureus, Streptococcus agalactiae,* rabbits

INTRODUCTION

World-wide, mastitis is one of the most important and costly diseases in dairy industry (Schepers and Dijkhuizen, 1991; Hortet and Seegers, 1998) that results in 35 billion dollars annual losses (Ratafia, 1987). National Mastitis Council Inc. of USA has reported that 70-80% milk losses are due to sub clinical mastitis (Philpot and Nickerson, 1991). Mastitis affects both quality (Urech *et al.*, 1999; Ullah *et al.,* 2005) and quantity (Arshad *et al.,* 1995; Rodostits *et al.*, 2006) of milk.

A number of microorganisms are responsible for causing mastitis including bacteria, fungi, yeasts and mycoplasma. But the most frequent pathogens associated with mastitis are bacteria such as *Staphylococcus aureus*, *Streptococcus agalactiae, Escherichia coli, Corynebacterium pyogenes*, *Streptococcus dysgalactiae* and *Streptococcus uberis* (Radostits *et al.*, 2006). The most prevalent pathogens in Pakistan are contagious pathogens including *S. aureus* and *Str. agalactiae* that account for about 75% of total mastitogens prevalent in Pakistan followed by environmental pathogens especially *E.* coli (Allore,1993). Because of the association of different microorganisms with mastitis, a vaccine with more than one organism would conceivably be more pragmatic (Hill, 1990; Calzolari *et al.*, 1997; Giraudo *et al.,* 1997; Yancey, 1999).

In Pakistan, because of extremely small herd size (more than 80% animals kept in group of 3-4 animals/family; (Jost, 1980; Tuefel, 1998), widely rampant poverty and illiteracy, lack of any milk quality premium program, the standard mastitis control practices (e.g. post milking antiseptic teat dipping and dry period antibiotic therapy) as advocated by the National Mastitis Council Inc, USA (Nickerson, 1994) are conceivably difficult to be adopted. Even on well-organized private dairy farms and those in public sector mastitis control is not in place. Against this backdrop, vaccination against the common mastitis pathogens holds the promise of an adjunct/alternative mastitis control strategy (Koiranen, 1977).

Adjuvants are modulators of immune system and include a range of substances. Among the several adjuvants alum adjuvants are safe and most commonly used in human and veterinary medicine (Edelman, 1992). It can be used as a component of a mastitis vaccine (Sears and Belschner. 1999). The present paper describes the efficacy of bivalent aluminium hydroxide adjuvanted mastitis vaccine in locally bred rabbits.

MATERIALS AND METHODS

Isolation, identification and bio-characterization of vaccinal organisms

For the isolation of vaccinal *S. aureus* and *Str. agalactiae,* aseptically collected milk samples (n= 95) from acute mastitis in buffaloes were cultured on blood agar (containing 5% sheep blood), Staph. 110 medium (Oxoid, Ltd, UK) and Edward's medium as per the methods described by National mastitis Council, Inc. USA (1990). Gram-positive, catalase-positive α and β hemolytic coccal isolates, presumptively identified as staphylococci, were subjected to coagulase test using rabbit plasma (at 4 hours) for determination of coagulation property of organism. Biotyping of the isolate was determined by using a commercial kit (api-STAPH; BioMerieux, France). Latex Slide Agutination test using Staphytect plus kit (Oxoid, Ltd, Basingstoke, Hampshire, UK) was conducted for the determination of clumping factor, protein A and certain polysaccharides found exclusively in *S. aureus.* Gram-positive, catalase-negative, β hemolytic coccal isolales presumptively identified as streptococci were further grown on Edward's medium for confirmation. These isolates were subjected to CAMP test, Esculin test and API- 20-Strep kit (BioMerieux, France).

Determination of expression of pseudocapsule of *S. aureus* cultures

An auto-agglutination procedure described by Watson and Watson (1989) was used for the confirmation of expression of pseudocapsule (a cardinal virulence and immunological factor).

Pathogenecity, antibiotic sensitivity and immunogenecity testing of vaccinal *S. aureus* and *Str. agalactiae*

Pathogenecity of *S. aureus* and *Str. agalactiae* was conducted in rabbits by subcutaneous inoculation of 0.2ml of bacterial suspensions containing 10^6CFU/ml. Each suspention was inoculated separately. The morbidity/ mortality was observed for 24-40 hours.Disc diffusion method on Muller-Hinton medium was used to determine the antibiotic susceptibility profiles of the vaccinal organisms following the guidelines of National Committee for Clinical Laboratory Standards (NCCLs, 1994; NLLCs, 2001). *Staphylococcus aureus* ATCC 25923 was used as a sensitive quality control. These isolates were then maintained at -20^{0}C in trypticase soy broth (Difco Labs., Detroit, Michigan) containing 20% glycerol until needed (Muhmmad, 1992). Fifteen adult healthy local bred rabbits were randomly selected and divided into three equal groups (viz. G_A and G_B and G_C) of five rabbits each. The concentration of *S. aureus* and *Str. agalactiae* antigenic preparation was set at 10^6 cells mL^{-1} of the organism using Breed-smear and spectrophotometric methods (Awan and Sajjad-ur-Rehman, 2002; Athar, 2007). The rabbits of group G_1 were dosed with *S. aureus* antigen @ 0.2 mL subcutaneously (SC) twice at weekly interval. The rabbits of group G_2 were given *Str. agalactiae* antigen while rabbits of group G_3 were kept as control receiving placebo (PBS). Blood samples were collected at weekly intervals for 3 consecutive weeks from inoculated rabbits and sera harvested for determination of the antibody response through indirect haemagglutination test (Tamura *et al.*, 1985; Butt, 2006 and Athar, 2007).

Preparation of aluminium hydroxide adjuvanted *S. aureus* and *Str. agalactiae* mastitis vaccine

Preparation of sterile whey

Whey was prepared by rennin precipitation of bubaline milk samples. Five mg of rennin was dissolved in 270 mL of saline and one mL of this solution was added to 10 mL delipidized buffalo milk. After 30 minutes at 37°C, the samples were centrifuged at 10,000 × g for 20 minutes. The whey samples were then sterilized by passing through a 45 μm filter paper (Millipore Corporation, Bedford, MA, USA). Whey was also required to provide the growth factors to *Str. agalactiae* (Brown and Baetz, 1976; Butt, 2006 and Athar, 2007).

Inactivation and preservation of dead stock cultures

The pure suspension of individual cultures of *S. aureus* and *Str. agalactiae* were treated with 0.4% formalin v/v for 12 hours and preserved under refrigeration temperature for further use. Prior to inactivation, blood agar plates were used for determination of the purity of individual cultures (Giraudo *et al.*, 1997).

Dose adjustment and formulation of adjuvanted bivalent mastitis vaccine

The required concentration 5× 10^{10}, bacterial cells per milliliter of each *S. aureus* and *Str. agalactiae*, were adjusted streptophotometrically according to the methods described by Hirsch and Strauss (1964) and Awan and Rehman (2002) and vaccine was prepared as per the methods described by Watson and Davies (1993), Giraudo *et al.,* (1997); Shoukat *et al.,* (1998) and Athar *et al.,* (2007). Sodium azide, thimerosal, and formalin were added as preservatives at final concentrations of 0.001% (w/v), 0.001% (w/v), and 0.4% (v/v), respectively.

Incorporation of crude toxin extracts of *S. aureus* and *Str. agalactiae*

For incorporation of crude toxin extracts, supernatant fluid was collected from 48-hour modified nutrient broth (nutrient broth supplemented with sterile bubaline whey

@ 10% v/v) cultures of *S. aureus* and *Str. agalactiae*. The supernatant was autoclaved (121°C; 15 psi) for 15 minutes. This preparation was then centrifuged at 6000 xg for 30 minutes at 4°C and the supernatant thus prepared added to the vaccine preparation at a concentration of approximately 5 mg of dry weight per dose.

Preparation of Aluminium hydroxide gel

Aluminium hydroxide [$Al(OH)_3$] gel was prepared and then added to vaccine as an adjuvant @ 3.5% w/v (Giraudo *et al.*, 1997)

Final Composition of aluminium hydroxide adjuvanted mastitis vaccine

Each 5 mL dose of bivalent vaccine contained 5×10^{10}, bacterial cells of the *S. aureus* and *Str. agalactiae* isolates, 1.5 mL of crude toxin extract, 0.02 mL formalin, 0.00005 g of thimerosal, 0.00005 g of sodium azide and PBS. Aluminum hydroxide (3.5%, wt/vol) was added as an adjuvant.

Quality control of aluminium hydroxide adjuvanted *S. aureus* and *Str. agalactiae* mastitis vaccine and its evaluation in rabbits

Samples of vaccine were assayed for sterility, safety, stability and challenge protection assay (Griffin, 1979; Giraudo *et al.*, 1997; Butt, 2006; Athar, 2007).

Determination of humoral response to aluminium hydroxide adjuvanted *S. aureus* and *Str. agalactiae* mastitis vaccine

In this experiment, 10 adult locally breed healthy rabbits were divided into two equal groups (G_A and G_B). The rabbits of group G_A were inoculated were with aluminium hydroxide adjuvanted *Staphylococcus aureus* and *Streptococcus agalactiae* bacterin-toxoid where as the rabbits of G_B were kept as control dosed with all ingredient of vaccine but antigen. Blood samples were collected from inoculated rabbits and sera harvested for determination of the antibody response for 60 days through indirect haemagglutination test. The interval between primary and secondary vaccination was 15 days.

Data Analysis

Geometric mean titres were computed for both vaccinated and non vaccinated control groups and compared. Similarly, cumulative geometric mean titres were also determined. Antibiotics sensitivity profiles of *Staphylococcus aureus* and *Streptococcus agalactiae* were determined by measuring zone of inhibitions produced by different antibiotic discs and organisms were declared as sensitive, intermediately sensitive or resistant on the basis of zone of inhibition.

RESULTS

Isolation, identification and bio-characterization of vaccinal organisms

Isolation of *S. aureus* and *Str. agalactiae* was conducted following the procedures described by National Mastitis Council, Inc. USA (1990). Isolate which was Gram-positive, catalase-positive & coagulase positive coccal isolates, showing alpha and beta haemolysis, protein A (Plate 1), pseudocapsule and api STAPH biochemical numerical profiles of 6736153 (Plate 2) was selected as a vaccinal *S. aureus* isolate. Similarly, a Gram-positive, catalase-negative and CAMP test positive having beta haemolysis (Plate 3) was selected as vaccinal *Str. agalactiae* (Table 1). This isolate had API 20 Strep 7-digit biochemical profiles of 3462414 (Plate 4).

Determination of expression of pseudocapsule of *S. aureus* cultures

The selected vaccinal *S. aureus* grown in modified nutrient broth (nutrient broth supplemented with sterile bubaline whey @) 10% v/v) and modified brain heart infusion broth (MBHIB) revealed the presence of pseudo-capsule around the bacterial cells. Strength of NaOH (molar) effectively demonstrating the presence of pseudocapsule ranged from 0.001– 0.02M whereas NaOH concentrations of >0.03M inhibited the process of auto-agglutination. Agglutination test using (Oxoid, Ltd, Basingstoke, Hampshire, UK) was conducted for the determination of clumping factor, protein A and certain polysaccharides found exclusively in *S. aureus*

Pathogenecity, immunogenecity and antibiotic susceptibility profiles of vaccinal isolates

Pathogenecity of *S. aureus, Str. agalactiae* was checked in five rabbits, four out of five rabbits were killed. Postmortem findings were septicaemia, petechiation on intestinal serosal surface, straw coloured fluid in abdomen, swollen kidneys, congested lungs and heart. Geometric mean titres (GMT) to 10^6 cells per ml of the suspensions containing *S. aureus* and *Str. agalactiae* in rabbits is presented in Table 2. The antibiotic response was significantly higher in the post-booster samples collected at day-14 and day-21 for the selected isolates. Antibiotic susceptibility profiles of the *S. aureus* and *Str. agalactiae* selected vaccinal isolates have been shown in Table 3. Vaccinal *S. aureus* was sensitive to enrofloxacin, norfloxacin, chlortetracycline, lincomycin, tylosin, amoxicillin, cephalexin, clarithromycin and ceftiofur. This selected *S. aureus* vaccinal isolate was intermediary sensitive to

Table 1. Colony, and biochemical characteristics of vaccinal field isolates of *S. aureus*, *Str. agalactiae*, used in the preparation of vaccine

Characteristics	*S. aureus*	*Str. agalactiae*
Hemolytic pattern on blood agar	α and β	β hemolytic
Gram's staining	G +ve	G+ve
Microscopic picture of the organism in stained smear prepared from Broth culture	Cocci	Cocci in chain
Catalase test	+ve	-ve
CAMP Test	NA	+ve
Protein A, clumping factor and polysaccharides	+ve	NA
API-Staph profile Biomerieux (France)	6736153	NA
API-Strep-20 profile Biomerieux (France)	NA	3462414

Table 2. Geometric mean serum IHA antibody titres (GMT) against vaccinal isolates of *Staphylococcus aureus* and *Streptococcus agalactiae* antigen in rabbits injected twice at day 0 and day 7 in rabbits

Groups of rabbits	Day-1	Day-7	Day-14	Day-21
G_1	1.3	10.6 x (8.15)	34.3 x (26.38)	21.1 x(16.20)
G_2	1.2	8.6 x (7.16)	27.9 x (23.25)	17.1 x (14.25)
G_3	1.2	1.6	2.0	2.0

Table 3. Antibiotic susceptibility profiles (size of zone of inhibition in mm) of *S. aureus* and *Str. agalactiae* isolates as per NCCLS (2001)

Antibiotic disc	Disc concentration	*S. aureus*	*Str. agalactiae*
Amoxicillin	25ug	16 (S)	20 (S)
Cephalexin	30 ug	30 (S)	32 (S)
Ceftiofur	30 ug	32 (S)	40 (S)
Chlortetracycline	30ug	32 (S)	18 (I)
Enrofloxacin	5ug	26 (S)	22 (S)
Clarithromycin	15ug	24 (S)	26 (S)
Lincomycin	2ug	30 (S)	36 (S)
Metronidazole	5ug	0 (R)	0 (R)
Neomycin	30ug	16 (I)	20 (S)
Norfloxacin	10ug	26 (S)	16 (I)
Novobiocin	30ug	16 (R)	20 (S)
Pencillin G	10 units	0 (R)	0 (R)
Sulphamethoxazole/ Trimethoprim	23.75/1.25	0 (R)	18 (S)
Tylosin	15ug	26 (S)	26 (S)

NCCLS (2001)

neomycin and it was resistant to novobiocin, metronidazole, Pencillin G, and sulphamethoxazole/trimethoprim. Vaccinal *Str. agalactiae* was sensitive to amoxicillin, cephalexin, ceftiofer, enrofloxacin, linconycin, clarithromycin, neomycin, novobiocin, sulphamethoxazole/ trimethoprim and Tylosin. This isolate was intermediary sensitive to chlortetracycline and norfloxacin whereas it was resistant to metronidazole and pencillin G.

Preparation of aluminium hydroxide adjuvanted *S. aureus* and *Str. agalactiae* mastitis vaccine

In modified nutrient broth (nutrient broth supplemented with sterile bubaline whey @ 10%v/v) *S. aureus* expressed much abundant pseudocapsule and a better and faster growth was achieved for vaccinal *Str. agalactiae*. The final composition of aluminium hydroxide adjuvanted *Staphylococcus aureus* and *Streptococcus agalactiae* bacterin-toxoid is given in (Table 4).

Quality control of aluminium hydroxide adjuvanted *S. aureus* and *Str. agalactiae* mastitis vaccine and its evaluation in rabbits

A loopful of the vaccine was cultured onto blood agar and no growth was found in 48 hours, which indicated the sterility of vaccine under trail.The results of safety of vaccine in rabbits have been presented in Table 5. Mild to moderate swelling developed in response to subcutaneous administration of the higher (0.5 IM) dose of vaccine while 0.2 IM dose of the vaccine did not show any troublesome effect in the inoculated rabbits.

The results of stability of vaccine in rabbits have been presented in Table 6 and it was found that there was no change in syringe-ability, texture and color at 4ºC. With increase in storage time and environmental temperature some changes occur in color and texture of vaccine. None of the rabbits vaccinated with 2 shots of aluminium hydroxide adjuvanted *S. aureus* and *Str. agalactiae* bacterin-toxoid died till day 7 post

Table 4.Composition of Formalin-inactivated Aluminium hydroxide-adjuvanted Bivalent Vaccine (FABV)
Each 5mL of vaccine contained:

Staphylococcus aureus 6736153	5×10^{10} cells
Streptococcus agalactiae 3462414	5×10^{10} cells
Crude toxin extract	1.5 mL
Aluminium hydroxide gel	17.5 mg
Formalin	<0.02 mL
Thimeosal	0.00005 g
Sodium azide	0.00005 g
PBS	Q. S.

Table 5. Safety of aluminium hydroxide adjuvanted *Staphylococcus aureus* and *Streptococcus agalactiae* bacterin-toxoid in rabbits

Groups of rabbits	No. of rabbits	Dose/Route	Mortality	Morbidity No.	Morbidity pattern
G4	5	0.2 mL SC	0	0	-
G5	5	0.5 mL SC	0	1	Mild/Moderate (+)

Moderate/Severe: Swelling at the injection site which subsided within 2 days
(+) Elevation of rectal temperature 40 ± 0.5 °C on day-1 post inoculation

Table 6. Effect of temperature and duration of storage on the physical stability of aluminum hydroxide adjuvanted vaccine

Temperature	Days in Storage		
	7	90	180
4°C	Good	Good	Satisfactory
25°C	Good	Satisfactory	Satisfactory
37°C	Good	Good	Unsatisfactory

Good = Milky white suspension without any sedimentation
Satisfactory = Milky with a small portion of water phase temporarily separated which upon shaking readily becomes suspension
Unsatisfactory = Fails to become homogenous after shaking/change in color/sedimentation

Table 7. Challenge/protection Assay (vaccination*-challenge) using *S. aureus* (API Staph.6736153), *Str. agalactiae* (API-Strep 20 3462414) in vaccinated and unvaccinated groups of Rabbits**

Groups	Vaccination	No. of Rabbits			
		Total	Survived till day 7 post challenge	Dead within 7 days post challenge	% Survival
G_6	Bacterin-toxoid	5	5	1	100%
G_7	Unvaccinated control	5	1	4	20%

*2 inoculations of 0.2 mL each at 15 days interval
**Live inoculum containing 10^9 cfu/mL of vaccinal *S. aureus*, *Str. agalactiae* administered at day 30 post second inoculation

challenges with live inoculums of vaccinal *S. aureus*, *Str. agalactiae.* So, the percent survival of the vaccines was 100 while in control group percent survival rate was 20% (Table 7).

Serum antibody titres (GMT) against S .*aureus* were highest (78.8) at day 45 in the rabbits of group G_A which declined slightly to 73.3 on day 60. The gradual increase in IHA titre for *Str. agalactiae* was also observed at day 45 and day 60 post vaccination. Cumulative mean antibody titre (CMT) was 44.94 for vaccinal *S. aureus* isolate and 46.56 for vaccinal *Str. agalactiae* isolate while in control group (G_B) 2.12 CMT was recorded. The antibody response of vaccine in terms of geometric mean titres was significantly higher in vaccinated group at day 45 and 60 post vaccination as compared to control group (Table 10).

The study observations are also same as described by Butt (2006) and Athar (2007). The concentration of 10^{10} of *S. aureus* and *Str. agalactiae* was used in the present study. Opdebeeck and Norcross (1985) and Watson and Davies (1993) found that higher concentration above 10^{10} did not elicit a notable significant higher immune response in the experimental animals. An increase in concentration more than 10^{10} cell ml^{-} did not enhance the immune response but decrease in immune response may be observed. According to Watson and Davies "Doses of cells more than 10^{9} may give satisfactory results with different adjuvants formulation but it is clearly impractical to give > 10^{11} cell/ mL. Dose dependent immune response clearly indicated the immunogenecity for bacterial suspension. Butt (2006) and Athar (2007) clearly

Table 8. Geometric mean serum IHA antibody titres (GMT) against vaccinal *Staphylococcus aureus* antigen in rabbits at post vaccination days

Groups of rabbits	Day-0	Day-15	Day-30	Day-45	Day-60
G_A	2.0	14.9 x (7.45)	55.7 x (27.85)	78.8 x (39.4)	73.3 x (36.65)
G_B	1.6	2.0	2.0	2.5	2.5

Table 9. Geometric mean serum IHA antibody titres (GMT) against vaccinal *Streptococcus agalactiae* antigen in rabbits at post vaccination days

Groups of rabbits	Day-0	Day-15	Day-30	Day-45	Day-60
G_A	1.8	19.7 x(10.94)	59.7 x(33.17)	73.3 x(40.72)	78.3 x(43.5)
G_B	1.6	2.0	2.0	2.5	2.5

G_1 = Vaccinated group G_2 = Non-vaccinated group

DISCUSSION

Staphylococcus aureus, Streptococcus agalactiae, isolated organism were biocharacterized by following the practices as described by National Mastitis Council Inc., USA (1990). These procedures are in line with those of Watts and Nickerson, 1986; Gonzalez *et al.* (1989), Nikerson (1992), Chaudhary and Azam (1995), Fazal (1995) Butt (2006) and Athar, 2007. In the present study pathogenic *S. aureus* and *Str. agalactiae* clearly showed the antigenic potential with respect to primary and secondary dose at day 1 and 7 respectively. These findings are in line with Nisonoff (1985) who reported that secondary response is more intensive and effective as compared to the primary response because initial treatment with antigen may result in multiplication of responsive cells which may persist for a long time in animals after the treatment with antigen and that saves the animals from the infectious and non-infectious diseases. Recommendation of O.I.E Manual (Truszczynski and Blancou, 1996) also coincides with the present studies. stated that antigen dose 10^{10} for the preparation of vaccines is most suitable one.

Table 10. Cumulative mean serum IHA antibody titres (CMT) against vaccinal isolates in rabbits

Groups of rabbits	CMT against vaccinal isolates	
	S.aureus	*Str. agalactiae*
G_A	44.94	46.56
G_B	2.12	2.12

In the present study selected isolates of *S. aureus* and *Str. agalactiae* was sensitive to enrofloxacin, chlortetracycline, lincomycin, tylosin, amoxicillin, cephalexin, clarithromycin and ceftiofur followed by neomycin, chlortetracycline and norfloxacin.These isolates were resistant to novobiocin, metronidazole, Pencillin G, and sulphamethoxazole/trimethoprim. These study findings are comparable to some extent with that of Athar, 2007 who also reported about similar kind of trend in antibiotic susceptibility of these isolates. Contrarily Chaudhry and Azam (1995)

reported gentamicin to be the most effective *in vitro* antibiotic followed by chloramphenicol, kanamycin, oxytetracycline, cotrimoxazole, penicillin, doxycycline, ampicillin, and nystatin. This indicates haphazard use of certain antibiotics would have communiquered resistance in the present microorganisms and would easily lead to therapeutic failure. Therefore, use of antibiotics based on antibiotic susceptibility profile is highly warranted to achieving a better cure rate.

The selected vaccinal *S. aureus* isolate was grown on modified nutrient broth to get encapsulated growth. The autoagglutination method (Watson and Watson, 1989) was used to asses the extent of pseudocapsule elaborated by this organism. Microorganisms when ever grown inside the udder or *in vivo* produce a large, well-define capsule outside the wall (Watson and Watson, 1989). Only a few strains of *S. aureus* produce a true capsule, in general the organism is not encapsulated. However, when grown in vivo conditions, in a variety of lesions, *S. aureus* produces an extracellular glycocalyx comprised largely of hydrated polysaccharides. Pseudocapsule is neither a true capsule nor a slime layer. This glycocalyx increase virulence of the organism by impairing complement and antibody mediated opsonization and inhibiting phagocytosis (Karakawa and Young, 1979; Wilkinson *et al.*, 1979). Glycocalyx expression usually ceases on culturing S. aureus in vitro conditions in conventional bacteriological media (Watson and Watson, 1989). *Streptococcus agalactiae* also required enrichment of growth media. For this purpose, sterile bubaline whey was also added to nutrient broth for mass cultivation. It has been found that milk whey provide three stimulatory factors (F), denoted as F-1, F-2, F-3. F-2 and F-2 are cationic while F-1 is non-ionic. A mixture containing any two factors gives greater stimulation than either factor tested alone, and a mixture of all three gives the greatest stimulation. The F-2 activity was attributed to cystine (Brown and Baetz, 1976). Butt, 2006 and Athar, 2007 also used whey for enrichment of cultural media.

All the standard procedures recommended by ISO (1994), OIE (1996) and FAO (1998) were attempted for sterility and safety conformation of the experimental vaccine. For these preliminary studies, rabbits were used and it was observed that this vaccine is safe to inject and free of any side effects when given at the standard dose rate (0.2mL SC). However, a mild to moderate local swelling and increase in rectal temperature was noted when injected at a dose of 0.5 mL subcutaneously. Vaccination challenge studies in rabbits demonstrated that a significantly higher survival percentage was present in vaccinated rabbits as compared to control group. The outcome of the present study is in line with those of Giraudo *et al.* (1997) who also tested the safety of vaccine in rabbits and other laboratory animals. Butt (2006) and Athar (2007) also reported the similar kind of findings in their studies.

Rabbits receiving aluminium hydroxide bacterin-toxoid showed highest antibody titres (GMT) against *S. aureus* and *Str. agalactiae* as compared to control group. As far as *S. aureus* is concerned, the present study is in agreement with those of Han and Park (2000) who also reported a higher immune response at day 45 and 60 in rabbits receiving a vaccine containing aluminium hydroxide adjuvant. These finding are also similar to that of previous studies conducted by Butt, 2006 and Athar, 2007 in rabbits. They also noted higher increasing trend in antibody titres for *Str. agalactiae* at day 30, 45 and 60 similar trend was observed in present study against *Str. agalactiae*.

Conclusion: The results of the study indicated that the bivalent (*S. aureus* and *Str. agalactiae*) aluminium hydroxide adjuvanted vaccine was immunogenic in rabbits

REFERENCES

Arshad, M., F.K. Qamar, M. Siddique and S.T.A.K. Sindhu. 1995. Studies on some epidemiological aspects of bovine mastitis. In: Proc. National Seminar on Epidemiology of Livestock and Poultry Diseases. January 19-20, College of Veterinary Science, Lahore, Pakistan.

Athar, M. 2007. Preparation and evaluation of inactivated polyvalent vaccines for control of mastitis in dairy buffaloes. Ph.D. Thesis, Deptt. of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan.

Awan, J.A. and S.U. Rahman. 2002. Microbiology Manual. Uni-tech Communications, Faisalabad, Pakistan.

Brown, R.W. and A.L. Baetz. 1976. Separation from whey of three growth factors for *Streptococcus agalactiae*. Am. J. Vet. Res. 37: 75–78.

Butt, A.A. 2006. Evaluation of four adjuvanted trivalent vaccines for the control of mastitis in dairy buffaloes and cows. Ph.D. Thesis, Deptt. of Clinical Medicine and Surgry, University of Agriculture, Faisalabad, Pakistan.

Calzolari, A, J.A. Giraudo, H. Rampone, L. Odierno, A.T. Giraudo, C. Frigerio, S. Bettera, C. Raspanti, J. Hernandez, M. Wehbe, M. Mattea, M. Ferrari, A. Larriestra and R. Nagel. 1997. Field trials of a vaccine against bovine mastitis. 2. Evaluation in two commercial dairy herds. J. Dairy Sci. 80: 854-858.

Chaudhary, T.M. and M.W. Azam. 1995. Sensitivity of various organisms isolated from clinical cases of mastitis in cattle, buffalo and goats. Pak. J. Livestock Poul. 1: 74-76.

Edelman, R. 1992. An update on vaccine adjuvants in clinical trials. AIDS Res. Hum. Retrovirus, 8: 1409-1411.

FAO. 1998. Production Yearbook. Food and Agri. Organization, Rome, Italy.

Fazal-ur-Rehman. 1995. Studies on: (I) Evaluation of surf field mastitis test for the detection of subclinical mastitis in buffaloes and cattle, and (II) Antibiotic susceptibility of the pathogens. M.Sc. Thesis, Faculty Vet. Sci., Univ. Agri., Faisalabad, Pakistan.

Giraudo, J.A., A. Calzolari, H. Rampone, A. Rampone, A.T. Giraudo, C. Bogni, A. Larriestra and R. Nagel. 1997. Field trials of a vaccine against bovine mastitis. I. Evaluation in heifers. J. Dairy Sci. 80: 845–853.

Gonzalez, R.N., J.S. Cullor and D.E. Jasper. 1989. Prevention of clinical coliform mastitis in dairy cows by a mutant *Escherichia coli* vaccine. Can. J. Vet. Res. 53: 301–305.

Griffin, W.C. 1979. Emulsion: Encyclopedia of Chemical Technology. 3rd ed. Wiley Inter-Science Pub., New York, USA.

Han, H.R. and H.M. Park. 2000. Effects of adjuvants on the immune response of staphylococcal alpha toxin and capsular polysaccharide (CPS) in rabbit. J. Vet. Med. Sci. 62: 237–241.

Hill, A.W. 1990. Vaccinating against mastitis. Mastitis treatment–Making it work. Proc. 3rd British Mastitis Conf., Stoneleigh, UK, Oct. 17. pp. 49-55.

Hirsch, J.G. and B. Strauss. 1964. Studies on heat-labile opsonin in rabbit serum. J. Immunol. 92: 145–154.

Hortet, P. and H. Seegers. 1998. Loss in milk yield and related composition changes resulting from clinical mastitis in dairy cows. Prev. Vet. Med. 37: 1–20.

ISO. 1994. Quality management and quality assurance. Vocabulary, ISO8402; Geneva, Switzerland, Int. Org. for Standardization.

Jost, N.P. 1980. Livestock Production and Marketing Survey Punjab. Report # 5. Production and Sales of Milk and Ghee. LDC, LEP/AI and GTZ PN 74: 2041–2047.

Karakawa, W.W. and A.D. Young. 1979. Immunochemical study of diverse surface antigens of a *Staphylococcus aureus* isolate from an osteomyelitis patient and their role *in vitro.* J. Clin. Microbiol. 9: 399–408.

Koiranen, L. 1977. Significance of vaccination for prevention of mastitis. Suomen Elainlaakarilehti. 83: 77–78.

Muhammad, G. 1992. Staphylococci of bovine mammary gland: Conventional and molecular dynamics of infections, plasmid stability, reproducibility, and interspecific conjugal transfer of antibiotic resistance, Ph.D. Dissertation, Deptt. Vet. Preventive Medicine, The Ohio State Univ., Ohio, USA.

National Mastitis Council, Inc. 1990. Microbiological Procedures for the Diagnosis of Bovine Udder Infection. National Mastitis Council, Inc., Arlington, Virginia, USA.

NCCLS. 1994. Performance Standards for Antimicrobial Disk Susceptibility Tests, 5th Ed. Approved Standard M2-A5, National Committee for Clinical Laboratory Standards. Villanova, Pa, USA.

NCCLS. 2001. Performance Standards for Antimicrobial Susceptibility Testing. 11th Informational Suppl., NCCLS Document M 100-911 (ISBN 1-56238-426-0). National Committee for Clinical Laboratory Standards. Villanova, Pa, USA.

Nickerson, S.C. 1992. Vaccination attempts against inflammatory infections and mastitis control in heifers. In: Proc. Ontario, Vet. Med. Assoc. Conf; Hamilton, Ontario. pp. 293-297.

Nickerson, S.C. 1994. Progress in the development of mastitis vaccine. Proc. Ann. Mtg. National Mastitis Council, Inc., Arlington, USA. pp. 133–134.

Nisonoff, A. 1985. Introduction to Molecular Immunology. 2nd Ed., Sinaver Assoc. Inc., Massachusetts, pp. 183-200.

OIE, 1996. OIE Manual of standards for diagnostic tests and vaccines. 3rd Ed. Office Intl. Dairy Fed; Belgium.

Opdebeeck, J.P. and N.L. Norcross. 1985. Antibodies in bovine serum and lacteal secretions to capsular antigens of *Staphylococcus aureus.* Am. J. Vet. Res. 46: 1561–1565.

Philpot, W.N. and S.C. Nickerson. 1991. Mastitis: Counter Attack. Babson Bros. Co., Illinois, USA.

Radostits, O.M., C.C. Gay, K.W. Hinchcliff and P.D. Constable. 2006. Veterinary Medicine–A Textbook of the Diseases of Cattle, Sheep, Pigs, Goats and Horses. 10th Ed. W.B. Saunders Co., Philadelphia, USA.

Ratafia, M. 1987. Worldwide opportunities in genetically engineered vaccines. Bio-Technology (New York).

Schepers, J.A. and A.A. Dijkhuizen. 1991. The economics of mastitis and mastitis control in dairy cattle: A critical analysis of estimates published since 1970. Prev. Vet. Med. 10: 213–224.

Sears, P.M. and A.P. Belschner. 1999. Alternative management and economic considerations in *Staphylococcus aureus* elimination programs. In Proc. 38th Ann. Mtg. Natl. Mastitis Counc., Arlington, VA. Natl. Mastitis Counc. Inc., Madison, WI. pp. 86–92.

Shoukat, M., T.M. Choudhari and M. Ali. 1998. Production and evaluation of oil adjuvanted haemorrhagic septicaemia vaccine using Paraffin, Sunflower and peanut oil. Proc: Pakistan Congr. Zool. pp. 70.

Tamura, Y., H. Makie and S. Tanaka. 1985. An indirect hemagglutination test for the detection of antibodies to *Clostridium chauvoei*. Vet. Microbiol. 10: 315–324.

Tuefel, N. 1998. Village milk production in central Punjab (Pakistan). Buffalo Newsletter 10: 4–9.

Truszozynski, M. and J. Blancou. 1996. Office International Des Epizooties Manual of Standards of Diagnostic Tests and Vaccines. 4th Ed., 12 rue de prony, 75017 Paris, France.

Ullah, S., T. Ahmad, M.Q. Bilal, Zia-ur-Rehman, G. Muhmmad and S.U. Rehman. 2005. The effect of severity of mastitis on protein and fat contents of buffalo milk. Pakistan Vet. J. 25: 1-4.

Urech, E., Z. Puhan and M.S. Schalibaum. 1999. Changes in milk protein fraction as affected by subclinical mastitis. J. Dairy Sci. 82: 2402–2411.

Watson, D.L. and N.A. Watson. 1989. Expression of a pseudocapsule by *Staphylococcus aureus*: Influence of cultural conditions and relevance to mastitis. Res. Vet. Sci. 47: 152–157.

Watson, D.L. and H.I. Davies. 1993. Influence of adjuvants on the immune response of sheep to a novel *Staphylococcus aureus* vaccine. Vet. Microbiol. 34: 139–153.

Watts, J.L. and S.C. Nickerson. 1986. A comparison of the STAPH-Ident and STAPH-Trace systems to conventional methods in the identification of staphylococci isolated from bovine udders. Vet. Microbiol. 12: 179-187.

Wilkinson, B.J., P. Peterson and P.G. Quie. 1979. Infection and Immunity. Vet. Rec. 23: 502-528.

Yancey, R.J. 1999. Vaccines and diagnostic methods for bovine mastitis: fact and fiction. Adv. Vet. Med. 41: 2757-2773.