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EXPERIMENTAL TRIALS OF LIVE ATTENUATED AND INACTIVATED *STAPHYLOCOCCUS AUREUS* VACCINES IN RABBITS

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ABSTRACT

This study was conducted as a preliminary step on the rabbits for comparative efficacy of different vaccines of *Staphylococcus aureus*. Typical alpha-beta *Staph. aureus* species from a clinically affected mastitic buffalo was isolated. After proper identification based on cultural and morphological characteristics and API-Staph Trac system, a selected *Staph. aureus* isolate was used to prepare four different mastitis vaccines (Bacterin, oil-adjuvanted, dextran sulphate adjuvanted and live attenuated) after confirmation for pathogenicity and antigenicity, followed by its safety and sterility evaluation. Vaccines were tried in 25 rabbits divided into 5 equal groups. A separate vaccine was administered s/c @ 0.2 ml per animal and boosted at 15 days later. It was found that IHA antibody titers were higher (GMT 32-128) in live attenuated, dextran sulphate adjuvanted (GMT 32-128) and oil-adjuvanted (GMT 16-64) than the bacterin treated (GMT 16-32) group. All the vaccines showed an apparent immune response than the unvaccinated control group.

Key words: *Staph. aureus* vaccines, antibody titer, rabbits.

INTRODUCTION

A common practice in rural household is to keep 3–5 heads of buffaloes primarily for family use (Chaudhry, 1984). The animals are predominantly hand milked and contagious mastitis caused by *Staph. aureus* is most prevalent in Pakistan (Qamar, 1992; Fazal-ur-Rehman, 1995; Razzaq, 1998; Memon *et al.*, 1999). It seems important to find out its permanent remedial and preventive solution instead of recording its incidence and prevalence over and over again (Allore, 1993).

Keeping in view the dairy farming scenario prevailing in Pakistan, the present study was planned to prepare and compare the efficacies of four *Staph. aureus* vaccines (viz. live attenuated, plain bacterin, dextran sulphate adjuvanted bacterin and oil-adjuvanted bacterin) in rabbits as a first phase before their application in cows and buffaloes.

MATERIALS AND METHODS

Isolation of field isolate

A specific culture of a field isolate of *Staph. aureus* was obtained from the Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan. It was recultured and recharacterized on Staph 110 medium and Staph Track system (API-Staph Kit, USA), respectively.

Pathogenicity of selected vaccinal isolate of *Staph. aureus*

An activated growth (6-hours of incubation) of *Staph. aureus* from nutrient broth was adjusted to 10^6 cells per ml in phosphate bufferred saline (PBS) using McFarland standard. A single dose of 0.25 ml was administered s/c to four healthy adult rabbits. All the animals were kept under observation for 24 hours (Machant and Packer, 1983).

Optimization of cultural conditions

Optimal cultural conditions were standardized in order to get the encapsulated *Staph. aureus*. For this purpose, the selected field isolate was grown on blood agar (BA) plate, nutrient broth (NB) modified NB, brain heart infusion broth (BHIB), and modified BHIB. Modification of NB and BHIB was done by incorporating bubaline sterile whey @ 10% v/v. Extent of the expression of the pseudocapsule in different cultural conditions was confirmed and compared by using autoagglutination method (Watson and Watson, 1989).

Preparation of inactivated antigen

The culture suspension was examined for purity through Grams' staining method (Awan and Rahman, 2005). Total bacterial count was calculated spectrophotometrically (U-2001, Model 121-0032, Hitachi Instruments, Inc. Japan) at 640 nm wave length. After adding formaline (0.2%), the broth was kept for 12 hours at room temperature. Sterility of the suspension was assessed through cultural examination

on blood agar plate in next 24 hours and stored under refrigeration temperature for future use.

Evaluation of antigenicity in rabbits

Fifteen adult rabbits were divided into three equal groups. Rabbits of group R1 were inoculated with 0.2 ml of inactivated antigen through subcuataneous route as single dose. Group R2 rabbits were inoculated with 0.2 ml of inactivated antigen through subcutaneous route, followed by booster dose 7 days later. The rabbits of group R3 were kept as unvaccinated control. All the rabbits were maintained separately in the cages and given feed and water *ad-libitum*.

Serum samples were collected from each group randomly from three rabbits at weekly intervals for 21 days postinoculation. Antibody titers were measured using standard technique of indirect haemagglutination (IHA) test (Rahman *et al.*, 2005).

Evaluation of immune response

A total of 15 adult rabbits (weighing approx. 2.5 kg) were divided randomly into three equal groups (R4, R5, R6). Formaline inactivated antigen (*Staph. aureus*) was prepared in three different concentrations. The rabbits of group R4 were immunized with the formaline inactivated antigen preparation containing 10^8 cells/ml of *Staph. aureus*. The rabbits of group R5 were given preparation containing 10^{10} cells/ ml, while group R6 was administered with 10^{12} cells per ml. Serum samples were collected at weekly intervals for three consecutive weeks from rabbits of all groups. Antibody response to different antigen concentrations was evaluated through IHA test (Rahman *et al.*, 2005).

Extraction of crude toxin and vaccines preparation

For incorporation of crude extract, supernatant fluid was collected from 48-hour broth culture of *Staph. aureus*. The supernatant was separately autoclaved at 121°C for 20 minutes. This preparation was then centrifuged at 6000 x g for 30 minutes at 4°C and the supernatant was added to the vaccine preparation at a concentration of approximately 5 mg of dry weight per dose. Finally, the plain *Staph. aureus* vaccine was prepared (Giraudo *et al.,* 1997). Formaline inactivated *Staph. aureus* was adjusted to 10^{10} cells/ml spectrophotometrically at 640 nm wave length. Sodium azide was added as a preservative at final concentrations of 0.001% (w/v) and stored at 4°C for future use (Watson and Davies, 1993).

For dextran sulphate adjuvanted vaccine, dextran sulphate (DXS, sigma-Aldrich Co., USA) was added as an adjuvant in the plain vaccine at a final concentration of 50 mg DXS per ml for 5 ml dose (Watson and Davies, 1993). For oil-adjuvanted vaccine, liquid paraffin was used as an adjuvant, whereas Tween-80 and Span-80 were used as emulsifiers. Emulsification of oil and Span-80 was done at low speed (12000 rpm) with the help of ultra homogenizer (Ultraturex, USA). Antigen suspension was slowly added and continuously stirred at 180 × g in the ultra homogenizer at 4°C. Final oil emulsion vaccine was dispursed in sterilized glass vials with automatic cap seal system and stored at 4°C for future use (Shauket *et al.*, 1998).

For the preparation of live attenuated vaccine, an α-β haemolytic selected isolate of *Staph. aureus* was repeatedly passaged through culture on 5% sheep blood agar for 18th passages until it lost its haemolytic activity and then maintained in trypticase soy broth. This live attenuated isolate was grown for 24 hours in nutrient broth. Organisms were pelleted by centrifugation (3000 × g; 15 min.), washed (×2) with PBS (pH 7.2) and resuspended in sterile PBS. The concentration of bacteria was finally adjusted to 10^{10} cells/ml using spectrophotometric method (Watson and Lee, 1978).

Quality control

Sterility of vaccines was confirmed through cultivation of vaccine preparations on blood agar plate and incubating for 48 hours for the absence of any growth. Safety of live and killed vaccines was examined by s/c inoculation of 2 ml of the vaccines into eight rabbits (2 rabbits per vaccine).

To check the stability, sealed vials of inactivated *Staph. aurues* vaccines were kept at 4, 20 and 37°C till nine months. Physical properties of these vaccines were recorded. Live attenuated *Staph. aureus* was stored into three different media (peptone water, PBS and nutrient broth) to assess the length of viability. The viability was assessed by standard plate count method (Awan and Rahman, 2005).

Indirect haemagglutination test

Indirect haemagglutination test (IHA), already standardized by Rahman *et al.* (2005), was adopted for the evaluation of immune response in rabbits. Sonicated *Staph. aureus* antigen was used to sensitize sheep RBCs along with gluateraldehyde used as coupling agent. One percent sensitized sheep RBCs were finally used for conducting IHA test.

Immunogenicity trials

Twenty five adult healthy rabbits were divided randomly into five equal groups (R7 to R11). The rabbits of group R7 were vaccinated with live attenuated *Staph. aureus* vaccine. The rabbits of group R8 received plain bacterin. Rabbits of group R9 were given dextran sulphate adjuvanted vaccine. Rabbits of group R10 received oil-adjuvanted vaccine, whereas the rabbits of group R11 were kept as unvaccinated control. Serum samples were collected fortnightly for two months from the rabbits of all groups and antibody respose was evaluated through IHA test (Rahman *et al.,* 2005)

RESULTS

Biorecharacterization and pathogenicity of *Staph. aureus* isolate

The selected typical alpha-beta isolate of *Staph. aureus* showed the same 7 digit API-Staph biochemical profile 6336153 when it was biotyped again. All the

rabbits died within 18 hours and postmortem examination indicated septicaemia and patachiation on the intestinal serosal surface, straw-colored fluid in abdomen and swollen kidneys. Stained blood and straw colored smear indicated *Staph. aureus* infection.

Modified nutrient broth proved to be the best pseudocapsule developing medium in which autoagglutination was observed in molar concentration solution of sodium hydroxide starting from 0.04 to 0.001, while partial agglutination was found in modified brain heart infusion broth in molar concentration solution of sodium hydroxide between 0.04 and 0.001.

Antigenic response of *Staph. aureus*

There was an apparent primary immune response in group R1, which was 17.0 at day 14, followed by a slight decrease at day 21. In group R2, there was a distinct primary and secondary immune response that was 8.0 at day 7 and was maximum (26.0) at day 21. There was no such response in unvaccinated control rabbits.

Dose dependent immune response of *Staph. aureus*

The trend of antibody production (GMT) was about the same in three groups (R4 to R6). It was maximum (32.0) at day 14 in group R5 and remained almost consistent till day 21. It was less (21.1) in group R4 than that of group R6 in which antibody titer was 27.9 at day 14 and 21.1 at day 21.

The immunogenic response was highest in group R5 which was given the dose of 10^{10}/ml concentration, while it was the lowest in group R4. The immune response elicited by 10^{12} cells/ml concentration was in between the response shown by the other two concentrations i.e. 10^{8} and 10^{10}cells/ml. These results indicate that the rabbits of group R5 showed the best immune response and the concentration of *Staph. aureus* attained in this group was finalized in the preparation of attenuated and adjuvanted mastitis vaccines.

Immune response of four vaccines

In R7 group, antibody titers (GMT) were 32.0 at day 15 and 84.4 at day 30 with a constant increase until day 45 (128), followed by a slight decrease (119.4) at day 60. For group R9, GMT was 32.0 at day 15 and was followed by a sharp increase (128.0) at day 30 with consistency till day 45. There was a slight decrease in antibody titer at day 60 when it was 119.4. For group R10 vaccinated with oil adjuvanted *Staph. aureus* vaccine, GMT was 16.0 at day 15 and was followed by an upward trend, reaching 64.0 at day 30 and showed this consistency until the 60th day after primary vaccination.

In group R8 vaccinated with plain bacterin *Staph. aureus* vaccine, GMT was 16 at day 15 with a progressive trend to 32 at day 30 and it persisted till day 45, then it showed a downward trend reaching 21.1 at day 60. There was no such trend in rabbits of unvaccinated control group. It is apparent that dextran sulphate adjuvanted *Staph. aureus* vaccine attained the higher peak antibody titer than that of live attenuated *Staph. aureus* vaccine, the latter showed similar antibody titer during the last 15 days of trial as that of live attenuated *Staph. aureus* vaccine. Live attenuated *Staph. aureus* vaccine acquired the highest antibody titer at day 45. A consistent sharp upward trend of antibody titer was observed from day 15 to 30 in dextran sulphate adjuvanted *Staph. aureus* vaccine.

DISCUSSION

After getting a typical bovine alpha-beta *Staph. aureus* on rebiotyping, the same 7-digit biochemical profile was observed. This is in line with the standard methods of identification of this organism adopted by several workers (Gonzalez *et al.,* 1989; Nickerson, 1992; Chaudhry and Azam, 1995). It proved its pathogenicity and antigenicity in rabbits, as has previously been observed by Farooq *et al.* (2004). A concentration of 10^{10} cells/ ml was found to be more immunogenic as compared to other concentrations tested. A concentration of 10^{12}/ml was immunosuppressive initially contrary to the other two concentrations. Therefore, an increase in concentration above 10^{10} cells /ml did not enhance the immune response, rather a decrease was observed. Several other workers (Opdebeeck and Norcross, 1985; Watson and Davies, 1993) conducted dose dependent trails of *Staph. aureus* in rabbits and concluded that a concentration above 10^{10}/ml did not elicit a significantly higher immune response.

In the present study, autoagglutination test proved its worth as a preliminary test in detecting the pseudocapsule of the vaccinal *Staph. aureus* which is in line with the findings of Watson and Watson (1989), who found that organisms grown inside the udder, or in nutrient broth supplemented with ovine, caprine or bovine milk whey, produced a large, well defined pseudocapsule outside the cell wall. *Staph. aureus* grown in the presence of milk whey should have a strong propensity to autoagglutinate and produce pseudocapsular material, which contained antigens in common with staphylococci grown *in vivo*.

A better response was elicited by rabbits given live attenuated *Staph. aureus* vaccine (LSAV). Watson and Prideaux (1979) concluded that following immunization with a live *Staph. aureus* vaccine, the organisms multiply in the skin or tissues and an abscess develops. In rabbits vaccinated with either of four vaccines or kept as control, the highest antibody titers against *Staph. aureus* were recorded in those given the vaccine containing dextran sulphate as an adjuvant (Dextran sulphate adjuvanted *Staph. aureus* vaccinie-DSAV). This is in agreement with the finding of Han and Park (2000).

In this study, *Staph. aureus* plain bacterin elicited lowest HIA antibody titer in the rabbits vaccinated with simple bacterin. The animals of group R8 receiving plain bacterin showed the less peak titers than the other three (LSAV, DSAV and OSAV), followed by a sharp

decline which is in line with the findings of Watson and Prideaux (1979).

Oil adjuvanted *Staph. aureus* vaccinated (OSAV) group (R10) gained the peak titer slowly, followed by a persistency. Watson (1987) and Nordhaug *et al.* (1994) ascribed it to the mineral oil component of the vaccine, which constituted a depot from which vaccine components were slowly released.

A higher titer at day 30, followed by sharp decline at day 5 and day 0 in rabbits of group R3 concur with the pattern of antibody titers in sheep. It has been observed that addition of dextran sulphate to the vaccine provoked significantly higher antibody response that sustained for a longer period in sheep (Watson, 1987; Watson and Davies, 1993).

In conclusion, *Staph aureus* vaccines were found safe and effective in stimulating the serum antibody titers against the vaccinal isolate. These four vaccines may be tried in other species i.e. cows and buffaloes to control *Staph. aureus* mastitis in these species.

REFERENCES

Allore, H. G., 1993. A review of the incidence of mastitis in buffaloes and cattle. Pakistan Vet. J., 13: 1-7.

Awan, J. A. and S. U. Rahman, 2005. Microbiology Manual. Unitech Communications, Faisalabad, Pakistan, pp: 47-52.

Chaudhry, A. R., 1984. Livestock Development in Pakistan. Science Technology and Development. Pakistan Council for Science and Technology, Islamabad, 3: 29–38.

Chaudhry, T. M. and M. W. Azam, 1995. Sensitvity of various organisms isolated from clinical cases of mastitis in cattle, buffaloes and goats. Pakistan J. Livestock Prod., 1: 74-76.

Farooq, A. A., S. A. Muhammad, A. Shakoor, A. Khan and C. S. Hayat, 2004. Anitgenic response of a live *Staph. aureus* vaccine in rabbits. Pakistan J. Life Soc. Sci., 2: 70-72.

Fazal-ur-Rehman, 1995. Studies on: (1) Evaluation of Surf field mastitis test for the detection of subclinical mastitis in buffaloes and cattle, and (ii) antibiotic susceptibility of the pathogens. MSc Thesis, Univ. Agri. Faisalabad, Pakistan.

Giraudo, J. A., A. Calzolari, H. Rampone, A. Ramone, A. T. Giraudo. C. Bogni, A. Larriestra and R. Nagel, 1997. Field trials of a vaccine against bovine mastitis. 1. 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 mutant *Escherichia coli* vaccine. Canadian J. Vet. Res., 53: 301-305.

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

Marchant, I. A. and R. H. Packer, 1983. Veterinary Bacteriology and Virology. 7th Ed., CBS Publishers and Distributors, Delhi, India.

Memon, M. I., K. B. Mirbahar, M. R. Memon, N. Akhtar, S. A. Soomro and P. Dewani, 1999. A study on the etiology of subclinical mastitis in buffaloes. Pakistan J. Agri., Agri. Eng. Vet. Sci., 15: 34-36.

Nickerson, S. C., 1992. Mastitis Control: New Horizons. In: 31st Annual Meeting, National Mastitis Council, Inc. Arlington, Verginia, USA, pp: 221-230.

Nordhaug M. L, L. L. Nesse, N. L. Norcross and R. Gudding, 1994. A field trial with an experimental vaccine against *Staph. aureus* mastitis in cattle. 2. Antibody response. J. Dairy Sci., 77: 1276-1284.

Opdebeeck, J. P. and N. L. Norcross, 1985. Antibodies in bovine serum and lacteal secretions to capsular antigens *of Staph. aureus*. Amer. J. Vet. Res., 46: 1556-1561.

Qamar, F. K., 1992. Studies on some epidemiological aspects of bovine mastitis in Gujrat District. MSc Thesis, Univ. Agri., Faisalabad, Pakistan.

Rahman, S. U., M. Athar, A. Shakoor, G. Muhammad and A. A. Butt, 2005. Standardization of indirect haemagglutination test for titration of antibody against *Staphylococcus aureus, Streptococcus agalaetiae* and *Escherichia coli* isolated from bubaline mastitis. Int. J. Agri. Biol., 7: 441-444.

Razzaq, A., 1998. Comparative efficacy of Vetimast, Tetra-Delta, and Akamycin-D in mastitis of buffalo in and around Lahore. MSc Thesis, College of Vet. Sci., Lahore, Pakistan.

Shauket, M., T. M. Chaudhary and M. Ali, 1998. Production and evaluation of oil adjuvanted haermorrhagic septicaemia vaccine using paraffin, sunflower and peanut oil. Proc. Pak. Congr. Zool., 18: 255-261.

Watson, D. L. and C. G. Lee, 1978. Immunity to experimental staphylococcal mastitis:comparison of live and killed vaccines. Aust. Vet. J., 54: 374-378.

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

Watson, D. L. and J. A. Prideaux, 1979. Comparisons of *Staph. aureus* grown *in vitro* or *in vivo*. Microbiol. Immunol., 23: 553-574.

Watson, D. L., 1987. Serological response of sheep to live and killed *Staph. aureus* vaccines. Vaccine, 5: 275-278.

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