Pakistan Vet. J., 2006, 26(3): 115-117.

PRIMARY AND SECONDARY IMMUNE RESPONSE TO FORMALIN INACTIVATED *STREPTOCOCCUS AGALACTIAE* ISOLATES IN RABBITS

M. ABUBAKAR, G. MUHAMMAD[1] AND K. IBRAHIM

National Veterinary Laboratories, MINFAL, Islamabad,
[1]Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan

ABSTRACT

The present study was conducted to evaluate primary and secondary immune response to formalin inactivated *Streptococcus agalactiae* isolates in rabbits. *Streptococcus agalactiae* was isolated from mastitic buffaloes. The organism was characterized on the basis of morphological, cultural and biochemical tests. An increased geomean antibody titer was observed in rabbits inoculated with single dose (Group A) and double dose (Group B) of *Streptococcus agalactiae* antigen. It was also evident from the results that the double dose of *Streptococcus agalactiae* antigen in rabbits (Group B) showed better and long lasting humoral antibody response as compared to single dose (Group A).

Key words: Immune response, *Streptococcus agalactiae,* rabbits.

INTRODUCTION

Mastitis is the most important and expensive disease of dairy industry (Allert, 1995). It also causes huge economic losses to the dairy farmers due to decrease in milk production. Despite annual production of over 27 million tonnes of fresh milk by 22.7 million cattle and nearly 24 million heads of buffaloes, Pakistan is facing an acute shortage of milk supplies in major urban cities and approximately 16 million Dollars worth of dry milk is being imported every year (Economic Survey, 2003-04).

Because of rampant poverty and illiteracy, standard mastitis control practices (e.g., pre and post milking antiseptic teat dipping and dry period antibiotic therapy) as recommended by National Mastitis Council Inc. USA (Nickerson, 1994) are quite difficult to be adopted in a country like Pakistan. Against this backdrop, vaccination holds the promise of an alternative mastitis control strategy.

The aetiology of mastitis is very complex because a large number of microorganisms are known to cause inflammation of the udder. Generally, well-recognized organisms responsible for mastitis are *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dys-agalatiae, Corynaebacterium pyogenes, Pseudomonas auroginosa* and *Escherichia coli* (Radostits *et al.,* 2000). *Streptococcus agalactiae* is the second most common mastitis pathogen in buffaloes after *Staph. aureus* (Razzaq, 1998).

Streptococcus agalactiae is highly contagious and obligate infection of mammary glands. It generally causes sub-clinical mastitis that can, therefore, be diagnosed in laboratory (Meiri-Bendek *et al.,* 2002). It remains on the epithelial surface, causing tissue damage during growth and multiplication and stimulating an inflammatory response. It is transmitted from quarter to quarter and from animal to animal by fomites, calves and milking machines or on the hands of milking man (Cullor *et al.,* 1990).

Poor management and sanitary conditions, failure of therapeutics and control measures like pre and post-milking udder disinfections are the factors that motivate to develop effective vaccines against mastitis. In order to evolve an effective vaccine to minimize mastitis in the buffaloes, it is mandatory to evaluate the antigenic responses to important mastitis pathogens in laboratory animals so that optimum antigenic dose of these organisms could be determined. The present study was designed to evaluate the primary and secondary immune response to formalin inactivated *Streptococcus agalactiae* antigen in rabbits.

MATERIALS AND METHODS

Isolation and biocharacterization of field isolates

Isolation and biocharacterization of bacterial isolates from 20 mastitic buffaloes was conducted following the procedures described by National Mastitis Council, Inc. USA (1990) in the Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan. The purified *Streptococcus agalactiae* isolate was preserved in trypticase soya broth (Difco Labs., Michigan, USA), containing 20% glycerol and kept at -20°C.

Preparation of formalin-inactivated *Streptococcus agalactiae* antigen

Selected *Streptococcus agalactiae* isolate was inoculated in 500 ml flask having nutrient broth

enriched with sterile bubaline whey (10%), obtained from the rennet precipitation of fresh defatted bubaline milk (Watson and Watson, 1989). It was kept on an orbital shaker at 60 rpm for 48 hours. After that formalin (0.4%) was added to kill the *Streptococcus agalactiae* isolate. The formalized isolate was kept for 24 h at room temperature for the proper action of the formalin. The killed organisms were harvested by centrifugation at 6000 x g for 1 hour at 4°C. Two washings with sterile PBS (pH 7.2) were done. The pellet thus obtained was re-suspended in sterile PBS. The concentration of *Streptococcus agalactiae* was adjusted at 1 x 10^9/ml by spectrophotometer (Opdebeeck and Norcross, 1985). The preparation was stored at 4°C until utilized. Sterility was checked by streaking a loopful of the antigen onto blood agar, MacConkey agar plates and thioglycolate broth and incubating for 24-48 hours at 37°C.

Immune response in rabbits

A total of nine adult healthy albino rabbits, divided randomly into 3 groups (A, B and C) containing 3 rabbits each, were utilized for assessing the primary and secondary immune response of *Streptococcus agalactiae* antigen. To the rabbits of groups A and B, 0.2 ml of the inoculum containing 1 x $l0^9$/ml cells of *Streptococcus agalactiae* was injected subcutaneously, whereas the rabbits of group C were kept uninoculated control. The rabbits of group B were given a booster dose at the dose rate of 0.2 ml/ rabbit at day 15 of the primary injection. Serum samples were collected at 15 days interval till day 60 to compare the primary and secondary response to *Streptococcus agalactiae* formalin-inactivated antigen. The antibody titer was determined by indirect haem-agglutination (IHA) method (Sawada *et al.,* 1981).

RESULTS AND DISCUSSION

Mastitis can be caused by physical or chemical agents but majority of the cases are infectious and usually caused by bacteria. *Streptococcus agalactiae* is one of the major causative agents of intramammary infections in dairy cows. The establishment of infection depends not only upon the toxigenic capacity of the infecting strain of *Streptococcus agalactiae* but also on the environmental conditions. This includes the degree of specific immunity which the host may have developed against toxic products of the bacteria.

The study was conducted to monitor the antigenic response of formalin inactivated *Streptococcus agalactiae* antigen in rabbits. The ultimate objective of the study was to evaluate the experimental vaccine in buffaloes, which are the actual hosts of the disease. *Streptococcus agalactiae* isolate was selected after studying the morphological and biochemical characteristics of isolates. The selected isolates of *Streptococcus agalactiae* when subjected to morphological and cultural examination, all showed that they were gram-positive cocci arranged in chains. The size of organism ranged between 0.5-1.5 um. These variants were non-motile and non-spore bearing. These isolated variants produced transparent, moist and dewdrop like colonies on blood agar and gave α and β haemolysis. The colony size ranged between 1-2 mm after 48 hours incubation. These finding are congruent with those described by Collier *et al.* (1998).

The selected isolate of *Streptococcus agalactiae* was also negative for Esculin and catalase tests but positive for CAMP and Sodium Hippurate test which is in complete alignment with the findings of Opdebeeck and Norcross (1985).

Indirect haem-agglutination (IHA) method was used for evaluating primary and secondary immune response of *Streptococcus agalactiae.* Reliability of the IHA test for the determination of IHA antibody titers had been advocated by Azam *et al.* (1991). The IHA technique is reliable, sensitive, quite accurate and extensively employed for measuring immunity.

Formalin inactivated *Streptococcus agalactiae* antigen was inoculated either as a single dose (Group A) or double dose (Group B) to determine the primary and secondary immune response to the antigen in rabbits. The IHA antibodies in Group A obtained their peak (GMT = 64) at day 30 post inoculation (PI) with gradual drop up to day 60 PI (Table 1). In case of Group B, a peak in geomean antibody titer was observed 45 days post booster which then gradually declined up to day 60 post booster with GMT of 64 (Table 1). These results are in line with the findings of Arshed (2002), who reported a peak in GMT during the 4^{th} week post-booster, which then gradually declined.

Indirect haem-agglutination antibody titers rose significantly at first week booster in group B, indicating antigenic potential of the organism. Nisonaff (1985) has described that the secondary immune response is more intense because the initial inoculation of antigen leads to multiplication of responsive cells, which may persist for a long time in the animal.

Based on the findings of the present study it was concluded that *Streptococcus agalactiae* isolate showed antigenic response in rabbits. The antigenic response was higher in the animals getting booster dose of *Streptococcus agalactiae* antigen as compared to the animals getting single dose. Thus, the *Streptococcus agalactiae* antigen isolated in the present study could be used to evaluate the immune response against mastitis buffaloes and could be an initial step for the preparation of successful mastitis vaccine.

Table 1: Geomean antibody titers (GMT) of rabbits inoculated with formalin inactivated S*treptococcus agalactiae* as detected by IHA test

Groups	No. of doses	Sample No. (Rabbit)	IHA antibody titers at post inoculation day				
			0	15	30	45	60
A	Single Dose	1	4	8	64	64	32
		2	4	16	128	64	16
		3	2	8	32	16	8
		GMT	3	9.8	64	39.4	16
B	Double Dose	1	2	8	64	64	32
		2	2	16	128	128	64
		3	4	16	128	256	128
		GMT	2.5	12.1	97	128	64
C (Control)	Un-inoculated	1	2	0	0	0	0
		2	0	0	0	2	0
		3	2	2	0	0	2
		GMT	1.5	0.6	0	0.6	0.6

REFERENCES

Allert, C., 1995. Mastitis vaccines: Alternative strategies for control of environmental mastitis. Large Anim. Vet., 50(3): 10-14.

Arshed, M. J., 2002. Studies on immunoprophylaxis of fowl cholera. PhD Thesis, Deptt. Vet. Microbiol., Univ. Agri. Faisalabad, Pakistan.

Azam, M. W., I. Hussain, M. Ashfaque, M. Siddique, T. Mahmood and M. T. Siddiqui, 1991. Comparative immunogenicity of sonicated *P. multocida* and formalized bacterin in rabbits. Pakistan Vet. J., 11: 120-122.

Collier, A. Balows and M. Sussman, 1998. Topley and Wilson's Microbiology and Microbial Infections. 9th Ed (2nd vol). Arnold, London, UK, pp: 271.

Cullor, J. S., J. W. Tyler and B. P. Smith, 1990. Disorders of the mammary glands: In: "Large Animal Internal Medicine". Bradford, P. S. (ed). The C.V. Mosby Co., Toronto. pp: 1047-1067.

Economic Survey, 2003-04. Economic Survey. Livestock Wing, Ministry of Food, Agri. Livestock, Islamabad, Pakistan.

Meiri-Bendek, I., E. Lipkin, A. Friedmann, G. Leitner, A. Saran, S. Friedmann and Y. Kashi, 2002. A PCR-based method for the detection of *Streptococcus agalactiae* in milk. J. Dairy Sci., 85: 1717-1723.

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

Nickerson, S. C., 1994. Progress in the development of mastitis vaccine. Pros. National Mastitis Council, Inc., Arlington, USA. pp: 133-134.

Nisonaff, A., 1985. Introduction to Molecular Immunology. 2nd Ed., Sinaver Assoc. Inc. Massachuesetts, USA.

Opdebeeck, J. P. and N. L. Norcross, 1985. Immunogenic properties of *Staphylococcus aureus* and *Streptococcus agalactiae* administered separately and in combination to lactating cows. Aust. Vet. J., 62: 114-116.

Radostits, O. M., C. C. Gay, D. C. Blood and K. W. Hinchcliff, 2000. Veterinary Medicine. 9th ed. W. B. Saunders Company Ltd. London, UK, pp: 563-565.

Razzaq, A., 1998. Comparative efficacy of Vetimast, Tetra-Delta and Akamycin-D in mastitis of buffaloes in and around Lahore. MSc (Hons.) Thesis, Deptt. Clinical Med. Surg., College of Vet. Sciences, Lahore, Pakistan.

Sawada, T., R. B. Rimler and K. R. Rhoades, 1981. Indirect haemagglutination test that uses glutaraldehyde fixed sheep erythrocytes sensitized with extract antigen for detection of *Pasteurella* antibody. J. Clin. Microbial., 16: 572-576.

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