INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
1560–8530/2007/09–1–133–135
http://www.fspublishers.org

Dose Dependent Antigenic Response to Formalin Inactivated *Streptococcus agalactiae* Isolate in Rabbits

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ABSTRACT

The present study was conducted to evaluate dose dependent immune response of *Streptococcus agalactiae* in rabbits. *S. agalactiae,* one of the major causative agents of mastitis, was isolated from mastitic buffaloes. The organism was characterized on the basis of morphological, cultural and biochemical tests. It was found that the rabbits which were given dose @ 0.8 mL of *S. agalactiae* (10^{-6} cells per mL) showed the highest Indirect Haem-agglutination (IHA) antibody Titers.

Key Words: Antigenic; Response to formalin; *Streptococcus agalactiae*; Rabbits

INTRODUCTION

Mastitis is the inflammation of udder regardless of cause. It is the most important and expensive disease of dairy industry. It also causes huge economic losses to the dairy farmers due to decrease in milk production (Allert, 1995).

Because of rampant poverty and illiteracy, standard mastitis control practices (e.g. pre & post milking antiseptic teat dipping & 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 back-drop, 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, S. dysagalatiae, Corynaebacterium pyogenes, Pseudomonas auroginosa* and *Escherichia coli* etc. (Radostits *et al.,* 2000).

S. agalactiae is highly contagious and obligate infection of mammary glands. It remains on the epithelial surface creating 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 vaccine against mastitis. In order to evolve an effective vaccine to minimize mastitis in the target species i.e. 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. *S. agalactiae* is the second most common mastitis pathogen after *Staph. aureus* (Razzaq, 1998).

This paper describes the comparative immune response to various doses of formalin-inactivated *S. agalactiae* antigen in rabbits.

MATERIALS AND METHODS

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

Preparation of formalin-inactivated *Streptococcus agalactiae* antigen. Selected *S. 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 & Watson, 1989). It was kept on an orbital shaker at 60 rpm for 48 h. After that formalin (0.4%) was added to kill the *S. agalactiae* isolate. The bacterial isolate was kept for 24 h for the proper action of the formalin. The killed organisms were harvested by centrifugation at 6000 x g for 1 h at 4°C. Two washings with sterile PBS (pH 7.2) were done. The pellet thus obtained was re-suspended in PBS. The concentration of *S. agalactiae* was adjusted at 1 x 10^9/mL by spectrophotometer. 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 h at 37°C.

Antigenic response to formalin-inactivated *Streptococcus agalactiae* in rabbits. A total of 12 adult healthy rabbits, divided randomly into 4 groups containing 3 rabbits each, were utilized in this study. The rabbits of groups A, B, C and D were used for evaluating the dose-dependent immune response to *S. agalactiae* antigen. The antibody titer was determined by indirect haemagglutination (IHA) method (Sawada *et al.,* 1981; Tamura *et al.,* 1985). Inocula containing 10^6 cells/mL of *S. agalactiae* were injected subcutaneously with the increasing dose ranging from 0.20 mL, 0.40 mL and 0.80 mL in group A, B and C, respectively. The rabbits of group D were kept as un-inoculated control. Serum samples were collected at weekly intervals for four consecutive weeks for Indirect Haem-agglutination (IHA) antibody titers to find out the optimum antigenic concentration/dose for *S. agalactiae* antigen.

RESULTS

The study was undertaken to evaluate the immune response of formalin inactivated *S. agalactiae* in rabbits. The *S. agalactiae* isolate was selected on the basis of cultural, morphological and bio-chemical characteristics. The isolate was G + ve cocci, non-motile, arranged in chains. On blood agar, it produced translucent round colonies, glistening with clear area around it and beta (β) pattern of hemolysis.

Bio chemically, the isolate was Catalase – ve, Sodium Hippurate test + ve, CAMP test + ve and Esculin test - ve.

Dose-dependent antigenic response to *Streptococcus agalactiae* antigen. Sera samples of animals of group A (dose@ 0.2 mL per rabbit) indicated progressive increase in GMT with maximum value of 39.4 at day 28 (Table I).

Group B (dose@ 0.4 mL per rabbit) was given a higher dose than group A and it showed increase in GMT with maximum value of 48.5 at day 28 (Table II).

Group C (dose@ 0.8 mL per rabbit) was given a higher dose than group B still it showed increase in GMT with maximum value of 97 at day 28, which indicated a positive dose dependent antigenic response of *S. agalactiae* (Table III); whereas, the rabbits of control group D showed no increase in titers (Table IV).

Table I. Results of Indirect Haemagglutination (IHA) test at 0, 7, 14, 21 and 28 day post-inoculation in sera of experimental Rabbits with dose rate of 0.2 mL of *Streptococcus agalactiae* (10^6/mL)

Groups	Sample (Rabbit)	No.	IHA titers at post inoculation day			
		0	7	14	21	28
A	1	2	16	32	32	64
	2	2	8	16	32	32
	3	0	8	16	16	32
	GMT	1.5	9.8	19.7	24.3	39.4

Table II. Results of Indirect Haemagglutination (IHA) test at 0, 7, 14, 21 and 28 day post-inoculation in sera of experimental Rabbits with dose rate of 0.4 mL of *Streptococcus agalactiae* (10^6/mL)

Groups	Sample (Rabbit)	No.	IHA titers at post inoculation day			
		0	7	14	21	28
B	1	2	8	16	32	64
	2	0	16	32	64	64
	3	2	16	32	32	32
	GMT	1.5	12.1	24.3	39.4	48.5

Table III. Results of Indirect Haemagglutination (IHA) test at 0, 7, 14, 21 and 28 day post-inoculation in sera of experimental Rabbits with dose rate of 0.8 mL of *Streptococcus agalactiae* (10^6/mL)

Groups	Sample (Rabbit)	No.	IHA titers at post inoculation day			
		0	7	14	21	28
C	1	2	16	16	32	64
	2	4	32	32	64	128
	3	2	32	64	64	128
	GMT	2.5	24.3	32	48.5	97

Table IV. Results of Indirect Haemagglutination (IHA) test at 0, 7, 14, 21 and 28 day post-inoculation in sera of Un-inoculated Control Rabbits

Groups	Sample (Rabbit)	No.	IHA titers at post inoculation day			
		0	7	14	21	28
D	1	2	0	0	2	2
	2	0	0	2	2	0
	3	2	4	0	2	0
	GMT	1.5	1.5	0.5	2	0.5

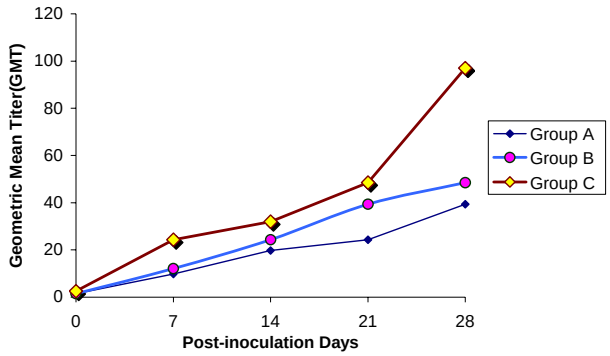
DISCUSSION

The ultimate objective of such studies is to evaluate the experimental vaccine in buffaloes, which are the actual hosts of the disease. However, preliminary trials are prerequisites before its final commencement in buffaloes. That is why present study was conducted in rabbits. *S. agalactiae* is one of the major causative agents of intramammary infections in dairy cows (Razzaq, 1998). The establishment of infection depends not only upon the toxigenic capacity of the infecting strain of *S. agalactiae* but also on the environmental conditions. This includes the degree of specific immunity, which the host may have developed against toxic product of the bacteria.

The positive milk samples (sub-clinical & clinical mastitis) were streaked on to Edward's medium. This medium is highly selective, for *S. agalactiae* as it inhibits the growth of all Gram negative bacteria and most of the Gram positive bacteria due to the presence of the Crystal violet and colonies of the *S. agalactiae* were purple colored. This was in accordance with the Hirsh and Zee (1999).

S. agalactiae isolate was selected after studying the morphological and bio-chemical characteristics of isolates. The selected isolates of *S. agalactiae* when subjected to morphological and cultural examination, all showed that

Fig. 1. Comparative immune response in group A, B and C against *Streptococcus agalactiae* antigen in rabbits



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 h incubation. These finding were congruent with those described by Collier *et al.* (1998).

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

Indirect haemagglutination (IHA) method was used dose-dependent assay of *S. agalactiae*. A total of 12 adult healthy rabbits, divided randomly in to 4 groups containing 3 rabbits each, were utilized in this study. The rabbits of groups A, B, C and D were used for evaluating the dose-dependent immune response.

Groups A, B and C resulted in a progressive increase in titers with maximum value at day 28 with GMT of 39.4, 48.5 and 97, respectively. While sera of group D (negative control) showed no increase in titers. This indicated a positive progressive dose dependent response of *S. agalactiae* isolate, which is in alignment to the findings of Tamura *et al.* (1985) but as we increased the dose above 1 mL, it resulted in tissue reaction as the site of deposit so it was preferred that the final dose should be 0.8 mL and not more than that (Fig. 1). This indicated that the selected field isolate is antigenic in nature and it produced dose dependent immune response in the laboratory animals, therefore, it is recommended for the preparation of successful mastitis vaccine.

CONCLUSION

1. The preparation of *S. agalactiae* antigen showed antigenic response in the experimental animals (rabbits).
2. The preparation was found safe and no untoward reaction was observed in any of the experimental animal.
3. The antigenic response to the preparation was dose-dependent.

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(Received 22 April 2006; Accepted 20 September 2006)