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Dose Dependent Antibody Response to Composite Formalin-inactivated *Staphylococcus aureus, Streptococcus agalactiae* and *Escherichia coli* in rabbits

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ABSTRACT

A total of 20 buffaloes clinically positive for mastitis were selected for the collection of milk samples. The bacterial isolates i.e. *Staphylococcus aureus, Streptococcus agalactiae* and *Escherichia coli* were isolated. At day 28, the geometric mean titer (GMT) of group D was 19.7, 16.0 and 32.0 for *S. aureus*, *Str. agalactiae* and *E. coli*, respectively. In group E the GMT at day 28 were 48.5, 39.4, and 48.5 for *S. aureus*, *Str. agalactiae* and *E. coli*, respectively. In group F the GMT at day 28 were 64.0, 64.0 and 78.8 against *S. aureus*, *Str. agalactiae* and *E. coli* respectively. The results of group D, E and F for assessing dose-dependent antigenic response showed that antigenicity of *S. aureus, Str. agalactiae* and *E. coli* was dose-dependent. There was direct relationship between antigen concentration and its antigenic response.

Key Words: Buffaloes; *Staphylococcus aureus*; *Streptococcus agalactiae; Escherichia coli*; antigenic response

INTRODUCTION

Inflammation within the mammary gland is designated, mastitis. Injury of any type to the internal tissues of the mammary gland leads to an inflammatory response or mastitis. Mastitis is a disease complex having different causes, different degrees of intensity and variations in duration and residual effects (Schalm *et al*., 1971).

Despite annual production of over 25.5 million tonnes of fresh milk by 22 million heads of cattle and nearly 23 million heads of buffaloes (Anonymous, 2000). Pakistan is facing an acute shortage of milk supplies in major urban cities and $ 16 million worth of dry milk is being imported every year (Siddiqui, 1999). Besides poor genetic potential, management and nutrition, sub-optimal health of milky animals particularly of milk producing organ (udder) i.e., mastitis is among the leading factors responsible for the shortfall of milk supply in Pakistan.

Because of extremely small herd size (more than 80% animal)was kept in herds of 3-4 animals per family (Jost, 1980; Teufel, 1998), widely rampant poverty and illiteracy and lack of any milk quality premium, standard mastitis control practices (e.g., post-milking antiseptic teat dipping and dry period antibiotic therapy) as recommended by the National Mastitis Council, Inc., USA (Nickerson, 1994) are conceivably difficult to be adopted in a country like Pakistan. In fact, these practices are totally non-existent even on well organized private dairy farms and those in the public sector (Military and government). Against this back drop vaccination shows the promise of suitable alternative mastitis control strategy in Pakistan in as much as it entails a single shot or a few at the most per year. In addition, it seems cost-effective.

A wealth of literature is available on the efficacy of mastitis vaccines in cows and ewes but reports regarding the efficacy of any kind of mastitis vaccine in buffaloes, the main stay of dairy industries in Pakistan and India are limited to a solitary report for a Staphylococcal vaccine (Pal & Pathak, 1977).

In order to evolve an effective vaccine to minimize the incidence of mastitis in the target species i.e. buffaloes, it is mandatory to evaluate the dose dependent antigenic responses to important mastitis pathogens in laboratory animals. The present study has been designed to evaluate the dose dependent antigenic responses to formalin-inactivated composite antigen preparation containing *S. aureus, Str. agalactiae,* and *E. coli,* the most prevalent mastitogens in Pakistan.

MATERIALS AND METHODS

Collection of milk samples. A total of 20 buffaloes clinically positive for mastitis were selected for the collection of milk samples. The milk samples were collected and processed in the Mastitis Research Laboratory, Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad (Pakistan) for the isolation and biocharacterization of mastitis pathogens following the procedures recommended by National Mastitis Council, Inc., USA (1990).

Isolation and biocharacterization of mastitis pathogens. The milk samples were streaked onto sheep blood agar plates. Morphological identification of primary growth was based on colony morphology, hemolytic pattern and Gram's

staining. Suspected colonies of *S. aureus, Str. agalactiae* and *E. coli* were further cultured onto Staph. 110 medium, Edward's Medium and MacConkey's agar plates, respectively to get pure isolates. Again the cultural examination was based upon colony color, colony morphology and, where relevant, the tests catalase, coagulase, casein hydrolysis, esculin, CAMP, Sod. Hippurate, triple sugar iron, methyl red and oxidase test.

Haemolytic properties. The purified field isolates of *S. aureus, Str. agalactiae* and *E. coli* were cultured on sheep blood agar to determine the haemolytic properties.

Preservation of bacterial isolates. The purified field isolates of *S. aureus, Str. agalactiae* and *E. coli* were preserved in Trypticase Soy Broth containing 20% glycerol at -20C (Muhammad, 1992).

Growth, inactivation and harvesting of bacterial isolates. The selected field isolates of *S. aureus, Str. agalactiae* and *E. coli* were grown separately in modified nutrient broth at 37°C on an orbital shaker at 60 rpm for 48 h. Expression of pseudocapsule of *S. aureus* was confirmed by auto-agglutination (Watson & Watson, 1989). Formalin (0.4%) was added to the broth culture for 24 h to inactivate the bacterial isolates. The inactivated organisms were harvested by centrifugation at 6000 *g* for 1 h at 4°C and two washings were given with PBS.

Antigen preparation. The composite antigen containing *S. aureus, Str. agalactiae* and *E. coli* was prepared (Opdebeeck & Norcross, 1982). Antigen preparation was made at three concentrations i.e. $1x10^7$, $1x10^8$ and $1x10^9$ cells/ml of each of *S. aureus, Str. agalactiae* and *E. coli* by Breed and Smear method (Awan & Rehman, 2002) and with the help of spectrophotometer (Hirsch & Strauss, 1964). The prepared composite antigen was stored at 4°C until used.

Sterility test. The sterility of prepared composite antigen was checked by streaking a loopful of composite antigen preparation onto blood agar, MacConkey's agar and thioglycolate broth.

Safety test. A 0.2ml and 1ml dose of prepared antigen was injected subcutaneously into three rabbits each to evaluate the safety of the composite antigenic preparation.

Antigenic Response.

Experimental animals. A total of 12 adult healthy rabbits were divided randomly into 4 groups (D, E, F and G) comprising of 3 rabbits each.

Inoculation of antigen. Inocula containing $1x10^7$, $1x10^8$, $1x10^9$cells ml^{-1} each of *S. aureus, Str. agalactiae* and *E. coli* were injected subcutaneously in rabbits of groups D, E and F, respectively. The rabbits of group G were kept as uninoculated control.

Serum collection. Blood samples were drawn aseptically from all the rabbits before inoculation and subsequently at weekly intervals till four consecutive weeks. Following collection the blood samples were centrifuged at 6000 *g* for 15 minutes and serum was collected in sterile plastic droppers. The serum samples were stored at -20C till further analysis.

Assay of serum antibodies. The level of antibodies in serum specific for *S. aureus, Str. agalactiae* and *E. coli* were assayed by Indirect Haemagglutination Test to find out the optimum antigenic dose of composite antigen. Finally the geometric mean titer was calculated (Brugh, 1978).

RESULTS AND DISCUSSION

A total of 20 buffaloes with clinical mastitis were selected for the isolation of *S. aureus, Str. agalactiae and E. coli*. The clinical mastitis was observed with the increased temperature, redness, edema, enlargement and hardness of the affected quarter, which is in accordance with Nickerson (1985) who reported same clinical findings of mastitis.

The *S. aureus* isolate showed that the organism was gram positive, cocci, arranged in form of clusters. This isolate was non-motile and produced yellow to golden colored colonies on Staph.110 medium having a diameter of 2-5mm. These finding were congruent with those described by Smith and Conant (1957), Cruickshank *et al*. (1975), Merchant and Packer (1983) and Quinn *et al*. (1994).

The selected isolate of *S. aureus* was also positive for catalase, Coagulase, and casein hydrolysis tests and also fermented the mannitol and produced complete haemolysis on blood agar. These findings were in complete agreement with Bell (1940) and Guidry *et al.* (1997) who classified the *S. aureus* on the basis of its biological and serological characteristics.

The selected *Str. agalactiae* isolate when subjected to morphological and cultural examination, it was gram-positive, cocci arranged in chains. The organism was non-motile and non-spore bearing. It produced transparent, moist and dewdrop like colonies on blood agar and gave ß haemolysis. The colony size was ranged between 1-2 mm. These finding were congruent with those described by Merchant and Packer (1983).

The selected isolate of *Streptococcus agalactiae* was also negative for esculin and catalase test but positive for CAMP and Sod. Hippurate test which was in complete alignment with Murphy (1959).

Escherichia coli isolate was also selected after studying the morphological and biochemical characteristics. The results revealed that *E. coli* was Gram negative, rod, produced pink color colonies on MacConkey's agar of diameter 3-5 mm. Further more *E. coli* was positive for catalase, triple sugar iron and methyl red test whereas negative for oxidase and citrate test. These findings were congruent with those described by Merchant and Packer (1983).

When we observed the dose-dependent antigenic response it was noted that there was gradual increase in antibody titre against *S. aureus, Str. agalactiae* and *E. coli* in all the animals of groups D, E and F. The maximum antibody titer was observed at day 28 with GMT 19.7, 16.0, 32.0 for *S. aureus, Str. agalactiae* and *E. coli*, respectively

Table I. Results of Indirect Haemagglutination (IHA) in rabbits of group D.

Group	Organism	GMT at day				
		0	**7**	**14**	**21**	**28**
D	***Staphylococcus aureus***	1.5	9.8	12.1	19.7	19.7
	Streptococcus agalactiae	1.5	4.9	9.8	16.0	16.0
	Escherichia coli	1.5	4.9	12.1	24.3	32.0

Table II. Results of Indirect Haemagglutination (IHA) in rabbits of group E.

Group	Organism	GMT at day				
		0	**7**	**14**	**21**	**28**
E	***Staphylococcus aureus***	0.8	6.1	16	24.3	48.5
	Streptococcus agalactiae	0.8	6.1	16	32.0	39.4
	Escherichia coli	2.0	4.9	19.7	39.4	48.5

Table III. Results of Indirect Haemagglutination (IHA) in rabbits of group F.

Group	Organism	GMT at day				
		0	**7**	**14**	**21**	**28**
F	***Staphylococcus aureus***	1.5	9.8	19.7	39.4	64.0
	Streptococcus agalactiae	1.5	12.1	32.0	64.0	64.0
	Escherichia coli	2.0	9.8	32.0	64.0	78.8

Table IV. Results of Indirect Haemagglutination (IHA) in rabbits of group G.

Group	Organism	GMT at day				
		0	**7**	**14**	**21**	**28**
G	***Staphylococcus aureus***	0.6	1.5	0	0.6	0
	Streptococcus agalactiae	1.5	0	0.6	1.5	0
	Escherichia coli	1.5	0.6	0	0.6	0.6

in group D. In animals of group E maximum antibody titer was observed at day 28 with GMT 48.5, 39.4 and 48.5 for *S. aureus*, *Str. agalactiae* and *E. coli*, respectively. In group F the maximum GMT was 64.0, 64.0 and 78.8 at day 28 for *S. aureus*, *Str. agalactiae* and *E. coli* respectively. When we compared the group D, E and F, the maximum antibody titer was found in group F followed by group E and D.

From these results it was concluded that antigenic response was dose dependent and there was direct relationship between antigen concentration and its antigenic response. Furthermore, it was observed that maximum antibodies were produced against *E. coli* followed by *S. aureus* and *Str. agalactiae.* These results were found in alignment with the findings of Opdebeeck and Norcross, (1982). The findings of the present study also correlate with the findings of Tamura *et al.* (1985).

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