Primary and Secondary Immune Response to Formalin Inactivated *Escherichia coli* Mastitis Isolate in Rabbits

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Abstract

Milk production is confronted by a variety of constraints, among these, mastitis is of prime importance. The study was performed to monitor the immune response of formalin inactivated *E. coli* preparation in rabbits. 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 the proper isolate and its optimum antigenic dose of these organisms could be determined. *E. coli,* 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. A total of nine (09) adult healthy albino rabbits, divided randomly into 3 groups (A, B and C) containing 3 rabbits each, were utilized. The antibody titer was determined by indirect haem-agglutination (IHA) test. To the rabbits of groups A and B, 0.2 ml of the inoculum containing 1×10^9 cells of *E. coli* mL^{-1} was injected subcutaneously whereas the rabbits of group C were kept un-inoculated 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 first injection. An increased geomean antibody titer was observed in rabbits inoculated with single dose (Group A) and double dose (Group B) of *E. coli* antigen. It was also evident from the results that the double dose of *E. coli* antigen in rabbits (Group B) showed better, sustainable and long lasting Humoral antibody response as compared to single dose (Group A). The results were also analyzed statistically using the single factor ANOVA and found significant difference among the groups.

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Introduction

Mastitis is recognized world wide as the most important and costly disease of dairy animals. Field surveys of major livestock diseases in Pakistan have indicated that mastitis is one of the most important health hazards in the country (Ajmal, 1990). Infectious agents like bacteria, viruses, fungi, and algae are mostly the primary causes of disease. Among these, the most important are bacteria, which could be divided into two classes: major pathogens *(Staphylococcus aureus, Streptococcus agalactiae, Corynebacterium pyogenes* and *coliform)* and minor pathogens (coagulase negative *Staphylococci* and *Corynebacterium bovis,).*

The incidence of clinical mastitis caused by environmental pathogens such as *Escherichia coli* is a concern of the dairy industry. The control of sub clinical mastitis with a subsequent reduction in milk somatic cell count (SCC) does not appear to decrease the incidence of clinical mastitis and may increase the susceptibility of cows to clinical mastitis caused by coliforms (Scott *et al.* 1998).

A sudden onset of a syndrome of pyrexia, muscular tremors of head and upper hind legs, ruminal stony and diffuse local swelling of the udder with straw colored watery non-odorous flaky secretion was in variably associated with isolation of *Escherichia coli* from the affected quarters of buffalo (Muhammad *et al.,* 1995).

Because of extremely small herd size [(more than 80% animal kept in herds of 3-4 animals/family (Teufel, 1998)], widely rampant poverty and illiteracy and lack of any milk quality premium, standard mastitis control practices (e.g., pre and 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 holds the promise of a 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.

In order to evolve an effective vaccine to minimize the incidence of mastitis in the target species i.e. buffaloes, it was mandatory to evaluate the antigenic responses to important mastitis pathogens in laboratory animals. *E. coli* is the third most common mastitis pathogen after *Staph. aureus* and *Strep. agalactiae.* The present study was designed to evaluate the primary and secondary immune response to formalin inactivated *E. coli* selected isolate 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. The purified *E. coli* isolate was preserved in trypticase soy broth (Difco Labs., Michigan, USA) containing 20% glycerol and kept at -20°C.

Preparation of formalin-inactivated *Escherichia coli* antigen

Selected *Escherichia coli* isolate was inoculated in a 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 *E. coli* isolate. The formalized 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 hour at 4°C. Two washings with sterile phosphate buffered saline (PBS, pH 7.2) were done. The pellet thus obtained was re-suspended in PBS. The concentration of *E. coli* was adjusted at 1×10^9/ 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 to Formalin-inactivated *Escherichia coli* 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 *E. coli* antigen. The antibody titer was determined by indirect haem-agglutination (IHA) method (Sawada *et al.,* 1981). To the rabbits of groups A and B, 0.2 ml of the inoculum containing 1×10^9 cells of *E. coli* mL^{-1} was injected subcutaneously whereas the rabbits of group C were kept un-inoculated 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 *E. coli* formalin-inactivated antigen. The titers were analyzed statistically using single factor ANOVA.

Results

The *Escherichia coli* isolate was selected on the basis of its morphological and biochemical characteristics. Morphology of *E. coli* isolate was G-ve rods and colony color was transparent (pinkish) on MacConkey agar.

Biochemical characters of *E. coli* isolate was catalase positive, lactose fermentation test positive, triple sugar iron test positive (Butt formation), potassium hydroxide test positive but Simmon's citrate test negative.

Primary and Secondary Immune Response to *Escherichia coli*

The Geo-mean titer (GMT) of sera samples of animals of group A showed progressive increase in antibody titers with reaching maximum at day 30 while the GMT of group B indicated a drastic increase in antibody titers even up to day 45 as a booster dose of antigenic preparation was given at day 15. While the sera samples of animals of group C showed no increase or decrease in antibody response (Table 1).

Table 1: Geomean antibody titers of rabbits inoculated with various doses of formalin inactivated *Escherichia coli* 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	2	32	64	32	16
		2	4	32	128	64	16
		3	2	16	64	32	16
		GMT	2.5	24.3	78.8	39.4	16
B	Double Dose	1	2	32	64	128	64
		2	4	32	128	128	256
		3	2	64	64	128	64
		GMT	2.5	39.4	78.8	128	97
C (Control)	Un-inoculated	1	0	2	0	2	0
		2	0	2	0	2	0
		3	2	4	2	0	2
		GMT	0.8	2.5	0.8	1.5	0.8

GMT=geo-mean titers

Discussion

Escherichia coli is the third most common mastitis pathogen after *Staph. aureus* and *Strep. agalactiae* with an over all prevalence of 8.96% in Pakistan but it is the most significant if u correlate it with the acute and per-acute mastitis (Razzaq, 1998).

The positive milk samples (sub-clinical and clinical mastitis) were streaked onto MacConkey's agar where it produced pinhead sized pink colored dry colonies with a central darker point as described by Cruickshank *et al.* (1975).
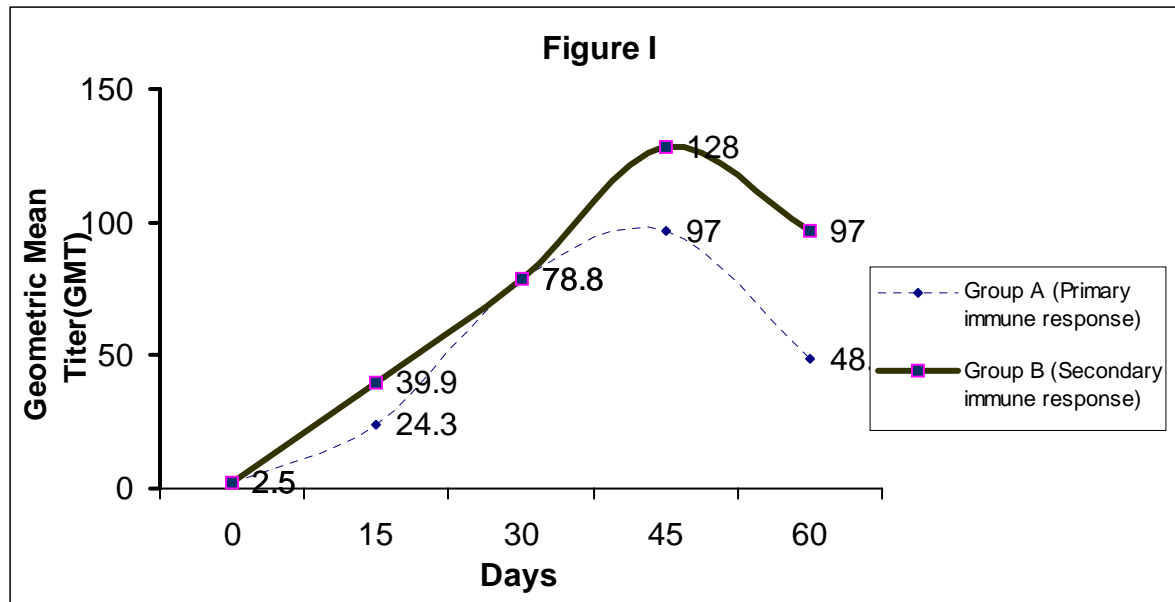
Indirect haem-agglutination (IHA) method was used for evaluating primary and secondary immune response of *E. coli*. It was important finding that formalin inactivated *Escherichia coli* antigen inoculated either as a single dose (Group A) or double dose (Group B) gave the IHA antibodies titers. The IHA antibodies in Group A obtained their peak (GMT=78.8) at day 30 post inoculation (PI) with gradual drop up to day 60 PI. In case of Group B a peak in mean IHA antibody titer was observed 45 days post booster which then gradually declined up to day 60 post booster with GMT of 97 but still this value was higher as compare to the peak of Group A (Fig 1). These results are in line with the findings of Arshed (2002) who reported a peak in GMT 4th week post-booster which then gradually declined.

A similar study was conducted by Vangroenweghe *et al.* (2004) to evaluate the dynamics of infection and the immunological response to varying numbers of *Escherichia coli* injected into the mammary glands of primiparous cows during the periparturient period. Primiparous cows have been shown to be more resistant to intramammary *E. coli* challenge, and an increase of the inoculum dose by 2 log10 units induced a more rapid clinical response and clearance of the organisms.

IHA antibody titers rose significantly at first week booster indicating antigenic potential of the organism which is in alignment with the findings of Nisonaff (1985) who 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, that also strongly concrete our isolate to be more immunogenic in nature and would be a right choice for vaccine production. The statistical analysis of these titers by ANOVA proved that there is a significant difference among the groups at the level $P<0.0001$ of significance (P-value= **2.17E-06**).

From the results it was concluded that inoculation of *E. coli* antigen showed antigenic response in rabbits. The response was significantly higher in animals given booster dose. It was also observed that the inoculation of *E. coli* antigen did not cause any untoward reaction in animals.

Figure 1. Comparative immune response in group A and B against *Escherichia coli* isolate in rabbits

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