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Evaluation of humoral immune response in cattle induced by the mastitis causing *E.coli* Biofilm vaccine in comparison with free cell vaccine by indirect Elisa

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Abstract
The study was carried out to evaluate humoral immune response with reference to serum IgG level by indirect ELISA, in mastitis causing *E. coli* Biofilm (BF) vaccinated and Free cell (FC) vaccinated groups. In all, eighteen cattle in early lactation which were free from mastitis were subjected to trials. Bentonite clay (an adjuvant by itself) based *E. coli* BF and FC vaccines were administered at 0, 30 and 60 days. Freund's incomplete adjuvant was incorporated in the first shot. Both BF and FC vaccinated groups showed significant difference with control group T cell proliferative immunological response by analyzing humoral immune responses indicated superiority of bovine mastitis causing *E.coli* BF vaccine over FC vaccine.

Keywords: Humoral immune, mastitis causing

Introduction
The Indian dairy industry has shown remarkable development in the past decade and is rapidly growing. Approximately 25% of the Indian agricultural gross domestic product is contributed by the livestock sector. *Escherichia coli* is also reported to be the most common etiological agent causing mastitis in cows following *Staphylococcus aureus* (Rajiv, 2006) [11]. Many problems are associated with antibiotic therapy like multiple etiology, drug resistance, non-co-operation of farmers and expensive therapy, delayed and unscrupulous use of antibiotics leading to improper treatment. Yet another most convincing hypothesis to explain failures with the therapy is the ability of many microorganisms to grow in biofilms (BF) in mammary gland, thus developing an innate resistance to almost all therapeutic agents. Biofilms are highly ordered microbial communities enmeshed in a carefully sculpted matrix designed for survival of organisms in multi or monogenus species in a specific micro niche. Interestingly, increasing evidence suggests that antibiotics also may stimulate the BF formation and sub inhibitory concentrations of antibiotics can also influence the expression of important bacterial virulence factors (Rachid *et al*., 2000) [10]. Furthermore, the use of antibiotics in the treatment of mastitis has created problems for milk processor and consumers.

In view of this, the present study was designed to evaluate the humoral immune response in cattle induced by the mastitis causing *E.coli* Biofilm vaccine in comparison with Free Cell vaccine by ELISA.

Material and Methods
***E. coli* culture:** For this study, *E.coli* O9 strain, which was isolated from the cases of bovine mastitis, typed at Central Research Institute, Kasauli and maintained at the Department of Microbiology, Veterinary College, Bangalore was used. *Escherichia coli* culture was routinely sub cultured and maintained on nutrient agar as per the standard procedures.

Analysis of outer membrane proteins (OMPs)
Extraction of outer membrane proteins
Outer membrane proteins were extracted from *E.coli* O9 serotype grown under following conditions:

a. Free cell: in 3 per cent TSB for 16 hrs
b. Biofilm cell: in 0.16 per cent TSB with 0.3 per cent bentonite clay for 7 days

The *E.coli* cultures grown under the above conditions were pelleted at 4000 rpm for 10 min at 4ºC. The pellet was washed three times in 10mM HEPES buffer and finally re-suspended in 10 mM HEPES buffer and stored at –20ºC until further processing.

Protein extraction and Quantification of proteins

Outer membrane proteins were extracted as per the methods of Bolin and Jensen (1987) [1]. The protein content in the OMP extract was estimated using a protein-dye-binding method according to Bradford (1976), using a readymade kit procured from *M/s* Bangalore Genei, Bangalore.

Procedure

Two ml of Bradford reagent was added to the aliquot of protein sample to be assayed. After ten min of adding the reagents, the absorbance was measured at 595 nm. Finally, the protein content was calculated by comparing the absorbance value with standard BSA ranging from 10 to 80 µg.

Preparation of vaccines

Two types of vaccines were prepared using *E.coli* O9.

Free cell/planktonic vaccine

The culture was grown in 3.0 per cent TSB for 16 hrs at 37°C and pelleted at 4000 rpm for 10 min. at 4°C. The pellet was washed thrice and finally resuspended in PBS to contain 10^9 cfu / ml after counting number of viable cells. The pellet was inactivated with 0.1 per cent formalin at RT for 24 hrs and stored at 4°C until use.

Biofilm vaccine

Seven-day-old BF cells grown in 0.16 per cent TSB, incorporated with 0.3 per cent bentonite clay were harvested by discarding the supernatant media to remove any FC. Bentonite clay with BF growth was adjusted to a final concentration of 10^9 cfu / ml with PBS after counting number of viable cells. The growth was inactivated with 0.1 per cent formalin at RT for 24 hrs and stored at 4°C until use.

Sterility test

The vaccine was inoculated onto nutrient agar, MacConkey agar and EMB agar in duplicates and incubated at 37°C and examined periodically for any bacterial growth up to seven days.

Immunization trials

Animals for trial

Eighteen cattle in early lactation which were free from mastitis were subjected to trials. Animals were grouped into three groups during the experiment. The approval of the Institutional Animal Ethics Committee was obtained. Of these 18, six cattle were maintained for each of the two *E. coli* O9 biofilm and free cell vaccines. Remaining six were controls. Prevaccinal sera and milk were collected from all the cattle.

Vaccination schedule

Six cattle each were immunized subcutaneously (S/C) by BF and FC vaccines of *E.coli* O9. The first dose in case of both FC and BF comprised of 5 ml suspension containing 4 $X10^9$ cells and 1ml of FIA. The subsequent two boosters were administered on days 30 and 60, which contained only $4X10^9$ cells in 5 ml without FIA. Details of vaccination are shown below in the Table 1.

Table 1: Details of different vaccine, dose and number of animals

E.coli 09 FC Vaccine	5 ml (4 $x10^9$ cells) with FIA	6 cattle
E.coli 09 BF vaccine	5ml (4 $x10^9$ cells) with FIA	6 cattle
PBS	5ml with FIA	6 cattle

Blood samples were collected on day 0 and then at an interval of every month upto end of the study. The sera samples from all the animals were subjected to ELISA to study Humoral immune responses.

Source of serum samples

Hyper immune sera

Hyper immune sera to *Escherichia coli* O9 FC and BF OMPs was the pooled post immunization sera collected after one month of the 2nd booster, which was preserved at -20°C. This was used as positive serum control (C+) in ELISA.

Healthy serum

Pooled pre-vaccinal sera collected from the cattle before the vaccination. This was used as negative control (C-).

Optimization of *Escherichia coli* O9 biofilm OMP antigen, Control sera and anti-bovine IgG HRP conjugate

Indirect ELISA was standardized by determining the optimum single working dilution of OMP antigen, Control positive and negative sera and Anti-bovine IgG HRP conjugate. It was performed by making various dilutions of antigen, control sera and conjugate as detailed below.

***Escherichia coli* O9 biofilm OMP antigen dilution**

A serial two-fold dilution of *Escherichia coli* O9 BF OMP antigen was prepared in carbonate-bicarbonate buffer (pH 9.6 ± 0.05) to provide 0.5µg protein/ml through 0.0625 µg protein/ml.

Serum dilution

A serial two-fold dilution of pooled pre vaccinal and post BF vaccinal sera collected after the second booster was prepared in 1% BG-PBST to provide a dilution of 1:100, 1:200, 1:400 and 1:800 dilutions.

Anti-bovine IgG HRP conjugate

Three dilutions of Anti-bovine IgG HRP conjugate, 1:20,000, 1:40,000 and 1:60,000 were prepared in 1% BG-PBST.

Checker board titration of *Escherichia coli* O9 biofilm OMP antigen, Control positive and Negative sera and anti-bovine IgG HRP conjugate

a) One hundred µl of antigen at various dilutions containing 0.5µg protein/ ml (in columns 1, 5 and 9); 0.25µg protein/ml (in columns 2, 6 and 10); 0.125µg protein/ml (in columns 3, 7 and 11) and 0.0625µg protein/ml (in columns 4, 8 and 12) were transferred to the respective wells along the columns of Maxisorp plate and incubated at 4°C O/N in refrigerator.
b) The content of the wells was discarded and the plate was washed three times with washing buffer and gently tapped over a tissue paper.
c) The unreacted sites on the carrier surface of the wells were blocked by incubating the plate with 100 µl of 1% BG-PBST at 37°C for one and half hour in the orbital shaker at 16 rpm.
d) The content of the wells was discarded and the plate was

washed three times as described in step b.

e) One hundred µl of pooled post BF vaccinal sera (Positive control:C+) at 1: 100 (in row A); 1:200 (in row B); 1:400 (in row C) and 1:800 (in row D) dilutions were added. Similarly, 100 µl of pooled pre vaccinal sera (Negative control:C-) at 1: 100 (in row E); 1:200 (in row F); 1:400 (in row G) and 1:800 (in row H) dilutions were added. The plate was incubated at 37°C for one hour in the orbital shaker at 16 rpm. The plate was washed as described in step b.
f) One hundred µl of Anti-bovine IgG HRP conjugate at various dilutions, including 1: 20,000 (in columns 1, 2, 3 and 4); 1: 40,000 (in columns 5, 6, 7 and 8) and 1:60,000 (in columns 9, 10, 11 and 12) in 1% BG-PBST were added and incubated at 37°C for one hour in the orbital shaker at 16 rpm. The plate was washed as described in step b.
g) One hundred µl of freshly prepared chromogen-substrate solution containing OPD and 3% H_2O_2 as substrate (4 µl / ml of chromogen) was added to each well and the plate was kept at room temperature for 15 min.
h) Finally, 50 µl of 2.5 N HCl was added to each well to stop enzyme-substrate reaction.
i) Absorbance values in control wells were read at 490 nm using software based Labsystems, Biorad ELISA reader with an interference filter at 492 nm.

Seromonitoring of *Escherichia coli* antibodies by ELISA

Vaccinal (post immunization) sera

Serum samples were collected from both *Escherichia coli* BF and FC vaccinated cattle at different intervals *i.e.*, pre-vaccinal, one month after first shot, a month after first and second boosters and then at monthly interval.

Protocol of indirect ELISA for measuring serum IgG response

The procedure of indirect ELISA used for assay of antibodies in bovine sera obtained from control, BF and FC vaccinated groups is described below.

a) An optimum single working dilution of *E.coli* BF OMP, containing 0.125µg protein/ml, was prepared in coating buffer (pH 9.6 ± 0.05) and 100 µl of this was added to each well. The plate was incubated at 4°C O/N in refrigerator.
b) The content of the wells was discarded and the plate was washed three times with washing buffer and gently tapped over a tissue paper.
c) The unreacted sites on the carrier surface of the wells were blocked by incubating the plate with 100 µl of 1% BG-PBST at 37°C for one and half hour in the orbital shaker at 16 rpm.
d) The content of the wells was discarded and the plate was washed three times as described in step b.
e) Conjugate control wells were maintained in wells A1B1 by adding 100 µl of 1% BG-PBST alone. Then, 100 µl of 1:400 diluted positive control (in wells C1D1) and the negative control (in wells E1F1) serum diluted in 1% BG-PBST were added. This was followed by addition of 100 µl of 1:400 diluted each test serum sample to wells in duplicate and incubated at 37°C for one hour in the orbital shaker at 16 rpm. The plate was washed as described in step b.
f) Then, one hundred µl of 1:60,000 diluted Anti-bovine IgG HRP conjugate in 1% BG-PBST was added to all the wells and then the plate was incubated at 37°C for one hour in the orbital shaker at 16 rpm and washed as described in step b.
g) One hundred µl of freshly prepared chromogen-substrate solution containing OPD and 3% H_2O_2 as substrate (4 µl / ml of chromogen) was added to each well and the plate was kept at room temperature for 10 min.
h) Finally, 50 µl of 2.5 N HCl was added to each well to stop enzyme-substrate reaction.
i) Absorbance values were read at 492 nm using software based Lab systems, Biorad ELISA reader with an interference filter at 492 nm. Readings were taken after the wells with only substrate-chromogen and HCl were blanked to 'zero'. Optical Density (OD) values were converted into Percent Positivity (PP) values by employing the formula,

$$\text{PP value of sample sera} = \frac{\text{OD value of sample sera}}{\text{OD value of C+}} \times 100$$

The present study was carried out to evaluate immunological response with reference to the bovine mastitis causing *E.coli* biofilm based vaccine in cattle. The immunological responses were assessed by evaluating the humoral immune response by indirect ELISA.

Results

Optimization of *E.coli* biofilm outer membrane protein antigen, Control sera and Anti-bovine IgG HRP conjugate

ELISA was standardized by adopting checker board titration, using *E.coli* O9 BF OMP antigen at 0.5µg protein/ml through 0.0625 µg protein/ml; Positive and Negative control sera were used at 1:100, 1:200, 1:400 and 1:800 dilutions and Anti-bovine IgG HRP used at 1:20,000, 1:40,000 and 1:60,000 dilution.

The PP values with respect to varying dilution of *E.coli* BF OMP antigen, control sera C+ and C- are shown in Fig.1. On plotting graphs, sudden drops in PP values was observed and ratio was highest between C+ and C- at 1:400 dilutions with 0.125µg protein/ml of antigen and 1:60,000 dilutions of Anti-bovine IgG HRP. At this sudden drop of PP values, ratio between the OD values of C+ and C- sera was maximum (2.9) (Fig.1) compared to other antigen dilutions. Hence, 0.125 µg protein/ml of *E.coli* O9 BF OMP antigen, 1:400 dilution of sera were used as optimum working dilutions for monitoring *E.coli* vaccinal antibodies in sera collected on days 0 (pre vaccinal), 30,60, 90, 120 and 150 from *E.coli* O9 BF and FC vaccinated and unvaccinated control groups. The details of optimization are furnished in Table 2.

Seromonitoring of post vaccinal antibodies in vaccinated groups

Serum samples were collected from both *E.coli* BF and FC vaccinated groups at different intervals, which include days 0, 30, 60, 90, 120 and 150. The average OD values of the serum samples collected at aforementioned periods were converted to PP values to obtain uniformity in results (Table 2 and Fig. 1).

Statistical analysis was made to compare the humoral immune response generated in all the three groups *viz.* control *E.coli* BF and FC vaccinated groups by using two-way ANOVA based on the PP values of serum samples collected at day

0,30,60,90,120 and 150. Analysis revealed, in the PP values of sera collected on day 30, 60, 90 120 and 150 from *Escherichia coli* O9 BF was significant (P<0.001) compared with the control (Fig. 2). Meanwhile, the PP values of sera collected on days 60 and 90 from *Escherichia coli* BF vaccinated group was found to be significant (P<0.01) when compared with the *Escherichia coli* FC vaccinated group (Fig. 2). Further, the PP values of the sera collected on day 120 and 150 indicated that *Escherichia coli* O9 BF vaccinated group showed significant difference in PP values (P<0.001) compared to the FC vaccinated group (Fig. 2).

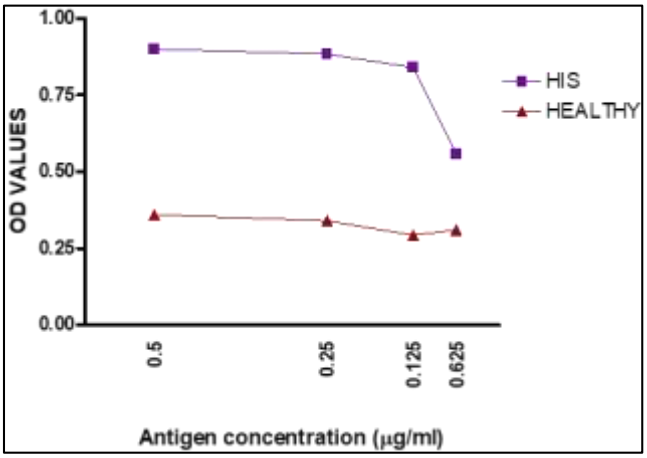

Fig. 1: Determination of optimum dilution of *E.coli* antigen (O9 BF OMP) for use in ELISA

Table 2: Standardization of indirect ELISA

	***E.coli* O9 biofilm OMP**
Dilution of Antigen	1:40 (0.125 µg per ml)
Average OD values with C+ at 1:400	0.84
Average OD values with C- at 1:400	0.29
Ratio of C+ and C-	2.89

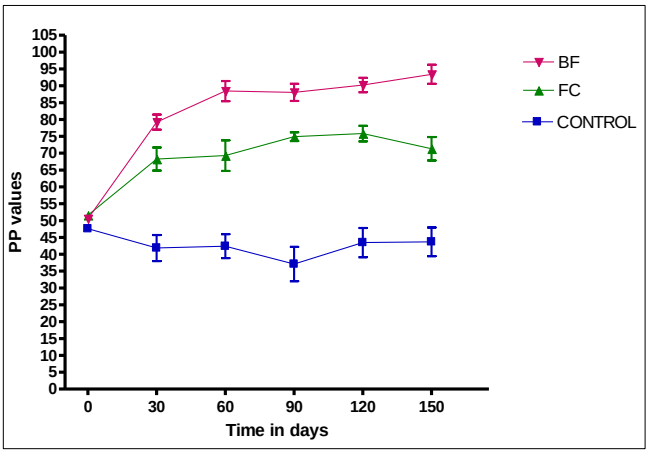

Fig 2: Distribution of PP values of serum samples from different group of Cattle

Discussion

India is the largest producer of milk in the world. The challenging task is the production of quality milk adhering to international standards to retain the first position in the global dairy industry. Bovine mastitis, an economically important disease of dairy cattle is considered to be a major challenge to the global dairy industry although several control strategies have been employed. Several authors have reported that *E.coli* was the most common etiological agent causing mastitis in cows following *Staphylococcus aureus* (Char *et al*., 1993 and Rajiv, 2006) [3, 11]. Nearly, 60 per cent of clinical mastitis cases are attributed to these environmental pathogens, especially *E.coli*.

In view of effective control of mastitis, immunization against mastitis has been a goal of researchers for many years and vaccination against mastitis pathogens is practiced in some dairy farms, especially in Western countries. Research on mastitis vaccines is being carried out since past 35 years and as a result, several mastitis vaccines are commercially available, including *E.coli* J5 core antigen based vaccines have been widely available in the United States. No such vaccines are available in India. The efficacy of such vaccines in reducing the severity of clinical disease has been demonstrated (Hogan *et al*., 1992) [5]. Further, the efficacy of conventional *E.coli* vaccines has not been proved in India. The ability of *E.coli* to form BF is considered to be a major virulence factor influencing its pathogenesis (Reisner *et al*., 2006). Recently, *E.coli* BF and FC based vaccines were compared by vaccination trials in rabbits (Kavitha, 2008) [7]. This study indicated the supremacy of *E.coli* BF vaccine as the CMT positivity, SCC values and percentage of quarter mammary gland quarters showing lesions on challenging, were significantly less in case of BF vaccinated groups. Further, serum IgG levels detected by ELISA was significantly high in BF vaccinated than FC vaccinated rabbits.

With this in view, to develop an effective vaccine and to control bovine mastitis caused by *E.coli*, the present study was undertaken to evaluate the immune response to bovine mastitis causing *E.coli* BF based and FC vaccines in bovines.

Enzyme Linked Immuno Sorbent Assay

Seromonitoring of the immune status of animals against mastitis causing pathogens has an immense value as a measure of level of protection. Furthermore, periodical monitoring of *E.coli* antibodies has a distinct advantage of providing an insight into the immune status against mastitis. In the present study, the antigen specific antibody production was measured by IgG indirect enzyme-linked immunosorbent assays (ELISA).

Optimization of *E.coli* outer membrane protein antigen

Indirect IgG ELISA was standardized at 0.125µg/ml of biofilm antigen, 1:400 dilution of positive serum control (C+) and negative serum control (C-) and 1:60,000 dilution of anti-bovine IgG-HRP conjugate. The end point titers of serum and conjugates were fixed at these values since at these values, there was sudden drop in the PP values with highest ratio (2.89) between the OD values of C+ and C- serum. Hence, 0.125µg/ml (1:40) dilution of *E.coli* BF OMP antigen, 1:400 serum and 1;60,000 Anti-bovine IgG HRP conjugate were used as optimum working dilutions for monitoring *E.coli* BF vaccinal antibodies in sera collected on days '0' (corresponded to prevaccinal) 30, 60, 90, 120 and 150 from control, *E.coli* O9 BF and FC vaccinated groups.

Serum IgG profile in control, BF and FC vaccinated groups

Statistical analysis was performed between the control, *E.coli* BF and FC groups by two way ANOVA based on the PP values of serum samples collected at days 0,30,60,90,120 and 150. There was a significant increase in the level of antibodies in *E.coli* BF vaccinated group on day 60 (P<0.001) and 90

(P<0.01) and further, significant increase (P<0.001) on day 120 and 150 compared with the FC vaccinated group was noticed. This data clearly indicated the presence of stronger antibody responses in the *E.coli* BF vaccinated group than *E.coli* FC vaccinated groups. However, IgG in the control group was found to be constant throughout the study. Both BF and FC vaccinated groups showed significant difference (P<0.001) with control groups. In a previous study by Hogan *et al*. (1997) [7], intramammary immunization using *E.coli* J5 mastitis vaccine enhanced IgG titers in serum and whey on day zero of lactation. Immunoglobulin G titers in serum also were greater at day 30 of the dry period and days 14 and 21 of lactation for cows that received intramammary immunization.

In the present work, the increased levels of IgG in the serum obtained from *E.coli* BF vaccinated is in agreement with Lee *et al*. (2005) [8], who reported the concurrent increase of the IgG levels in bovines following the immunization with *Staphylococcus aureus* trivalent vaccine. Increased levels of IgG antibody is required for promoting the bovine neutrophil phagocytes by performing opsonic activity (O'Brien *et al*., 2000). The role of IgG in protecting immunized cows has been attributed to the toxin-neutralizing abilities of IgG that bind to the core antigens of lipopolysaccharide (Tyler *et al*., 1992) [13].

Previous study on the BF and FC vaccines in Rabbits indicated the supremacy of BF vaccine as the CMT positivity, SCC values and percentage of mammary glands showing lesions on challenging, were significantly less in case of BF vaccinated groups. Further, serum IgG levels detected by ELISA was significantly high in BF vaccinated than FC vaccinated rabbits (Kavitha, 2008) [7].

Furthermore, the presence of IgG after primary and subsequent immunizations is an indicator of involvement of helper T -cells in the immune response and the induction of IgG producing B cells (Dresser and Popham, 1979) [4]. Thus, immunological memory is critical for rapid elimination and long lasting protection against mastitis (Tomita *et al*., 1995) [12].

Conclusion

Serum IgG level was significantly high in BF vaccinated than FC vaccinated and control cattle. *E.coli* BF vaccine was found to be superior to FC vaccine. Furthermore, the presence of IgG after primary and subsequent immunizations is an indicator of involvement of helper T -cells in the immune response and the induction of IgG producing B cells. Thus, immunological memory is critical for rapid elimination and long lasting protection against mastitis.

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