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BIOMARKER BASED DETECTION OF SUBCLINICAL MASTITIS BY LIQUID PHASE BLOCKING ELISA

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ABSTRACT

Mastitis is known to be an economically important disease hampering desired progress in the dairy industry. Mastitis caused by Streptococci is of the subclinical type.Early detection of mastitis is of paramount importance to avoid permanent damage to the udder and loss of milk.The presently detection methods of Electrical Conductivity test (EC) and Somatic Cell Count (SCC) for subclinical bovine mastitis are less reliable as the results are dependent not only on infection but changes also do occur due to various other stresses. Detection of etiological agent is cumbersome and time consuming and needed only for therapeutic purpose. Hence the development of a rapid and reliable test is the need of the hour. Detection of the molecules (Bio markers) that are produced during the invasion of the pathogen is the best approach. We developed a bio-marker based Liquid Phase Blocking ELISA for subclinical mastitis detection. A purified biomarker protein produced in E. coli and antiserum raised against it were used to develop the test (product patent filed-3807/DEL/2011) . A total of 87 bovine milk samples was collected from all lactating animals irrespective of the age at the lactation. Initial evaluation of SCC revealed that all the samples were from Subclinical Mastitis(SCM) except one, since, as per the conventional criteria the SCC values were more than >5 lakhsare considered to declare positivity. All the 87 samples were subjected to LPB ELISA and compared with SSC values. The results showed that the milk samples having SCC value 0.1 to 10 lakh showed average inhibition of 30% and samples with 10 to 15 lakh SCC showed the average inhibition value of 40% and samples with SCC 15 and above revealed an average inhibition of 50%. Liquid phase blocking ELISA (LPB-ELISA) developed by us showed high correlation with the SCC and represents a single test for the rapid detection of subclinical mastitis with good sensitivity, specificity.

KEY WORDS: Biomarker, Bovine milk, subclinical Mastitis, liquid phase blocking ELISA, detection method ,SCC.

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INTRODUCTION

Bovine mastitis (BM) is an inflammation of the mammary gland, usually due to a microbial infection (Watts, 1988), causing heavy losses to the dairy industry every year. These losses are primarily due to reduced quantity and of milk produced and sometimes, these losses are permanent in chronic BM. Therefore it is important to identify quickly the new clinical case in order to control infection in the herd. Mastitis is a complex multi etiological disease which can be simply defined as an inflammation of udder resulted from the introduction and multiplication of pathogenic microorganisms in the mammary gland. The causative bacteria can be classified as major or minor pathogens (Harmon, 1994). The suitability of a detection method for routine diagnosis depends on several factors, such as specificity, sensitivity, expense amount of time, and applied to large numbers of milk samples. The most common but unspecific method toidentify potential chronic infections is a somatic cell count and the California Mastitis Test in the diagnostic laboratory. It is important to identify the pathogen not only for antimicrobial therapy but also to monitor and control the rate of infection at the farm level. During the last 7 years; many tests have been developed for the diagnosis of BM. However, a rapid (less than 1 day), simple, and specific test for each kind of bacterium involved, has not been achieved. Many tests for the detection of pathogen in human mastitis are already excising. Some of these tests have been applied to pathogens of a bovine origin, such as the Minitek Gram- Positive test for *Streptococcus* (Watts, 1989), but with no success because of a lack of information on veterinary pathogens in the database. Enzyme-linked immunosorbent assay methods exist for *S. aureus*detection in cases of BM (Bourry&Poutrel, 1996), but the antibody titer does not correlate with the amount of infecting bacteria (Heisick*et al*., 1989 &Bourry&Poutrel, 1996). Other enzyme-linked immunosorbent assays were developed to screen milk for contamination with *Listeria* organisms (Adams *et al*., 1988 &Bourry*et al*., 1997). Most PCR used for the detection of microorganisms in milk or in other organic samples need a step of multiplying the bacteria in culture media (Andrews, 1983 &Thomas *et al.*, 1991 &Wernars*et al.*, 1991 & Lorenz *et al*., 1998;Queipo-Ortuno*et al*., 1999 &Reale*et al*. 1999; Khaled*et al*., 2010) and are, therefore, time-consuming. Rapid identification methods, in particular nucleic acid-based tests, have the potential to be extremely specific and can also discriminate between closely related organisms, such as *S. parauberis*and *S.uberis*. It has been previously shown that milk samples could serve as substrate for the amplification of specific DNA sequences using PCR (Lipkin*et al*., 1993 &Berri*et al*., 2000).

Subclinical mastitis does not lead to visible changes in the milk or udder; it is characterized by reduced milk yield, altered milk composition and the presence of inflammatory components and bacteria in milk. The main cellular defense mechanism includes macrophages, lymphocytes and in particular, neutrophils. After the invasion of the mammary gland, bacteria spread rapidly within the udder and have been found intracellularly within macrophages (Thomas *et al.*, 1994) although the impact of neutrophils in the control of invading *S. uberis*has been questioned (Thomas *et al.*, 1994). Though the mastitis is a major problem to the dairy industry, it can be cured, if detected early at sub-clinical level of suitable antibiotic therapy and can be controlled by following proper hygienic practices. However the major challenge is the detection of the infection in the udder at a very early stage or sub-clinical stage. Presently available methods include the tests to check the milk composition by counting SCCs, checking the milk pH and the presence of flocculations in the milk. However these are either unreliable or less sensitive. Detection of a pathogen can be considered as alternative methods. However in most cases they cannot detect at a very early stage of colony counting and enrichment of the organisms become

essential, which is time consuming and not feasible to handle several samples at a time. With the advent of molecular approaches to study the disease progression it has now become possible to develop suitable tests based on a biomarker approach or a genome detection approach. Hence there is an urgent need to conduct further research to develop a reliable and rapid test. Identification of host specificcytokineslike , IL8(Alluwaimi*et al.,* 2001,Riollet*et al*., 2000b, c; Shuster *et al.,* 1997., Persson Waller *et al*., 2003., HeliSimojoki*et al*., 2011), IL6(K. Hagiwara *et al*.,2001., Gabriela Trigo*et al.,* 2009.,Yoko Sakemi et al 2011)and protein factors like complement C3(Mueller *et al*., 1983., J.L. Boehmer*et al*.,2008.,Kenzo KAI et al 2002). L-selectin(Diez-Fraille*et al*., 2004), Pentraxin-3 (*PTX3*) (Ylva C *et al.,* 2008)and Component-3 of the proteose peptone (CPP3) (Kim et al 2011) thatis expressed during infection is an ideal approach to detect any pathological changes in the tissue. These biomarkers will help in providing the early warning to the disease. Based on this hypothesis we selected a set of bio-markers specific for mastitis, a synthetic gene carrying the antigenic sites was made and expressed in E. coli. The purified expressed protein and the antiserum raised against it were employed to detect the presence of specific biomarkers in the mastiticmilk using LPB ELISA. Thetest was evaluated with milk samples collected from mastitis positive animals and correlated with the SCC.

MATERIALS AND METHODS

Synthesis of Polyvalent Immunogenic Bovine Biomarker (PIBB)

The most immunogenic epitopes sequences of selected cytokines were identified and the gene was custom synthesized from the predicted, contiguously linked peptides with G-P-G bridges in between. The codon optimized gene was cloned in pET28a vector and expressed in *E. coli.* The protein was designated as POLYVALENT IMMUNOGENIC BOVINE BIOMARKER (PIBB)). (Patent filed with application number: 3807/DEL/2011).

Production and Purification of PIBB Protein in lab-scale fermenter.

The protein produced from transformed *E. coli*BL 21 (DE3) pLysusing standard procedures in the 1L fermenter (New Brunswick, USA). Briefly colonies harboring pET28a-PIBB were inoculated in 10 ml LB broth containing 50 µg/ml of kanamycin and incubated for 16 hrs at 37^0C in an orbital shaker. The overnight grown bacterial culture was used as a starter culture in fermenter containing 1.0 L synthetic medium (Sambrook J. and Russell D.W. 2006) without kanamycin. The culture was grown at 37^0 C with an agitation of 350 rpm. for 24 hr to reach an A_{600}30. The cell was induced for protein expression by the addition of is opropyl β- D-thiogalactopyanoside (IPTG) to a final concentration of 0.1 mM for 4 hr. The bacterial cells were harvested by centrifugation at 5000g and the pellet was suspended in 50 ml 10mMTris, 1mM EDTA pH 8.0 containing 10 mM Phenyl methyl sulphonyl fluoride (PMSF). The cells were lysed by one Freez-thaw cycle and the protein in the lysate supernatant was purified by affinity chromatography using Ni. NTA agarose (Qiagen, USA) using a standard protocol. A protein production was checked on 12% SDS-PAGE.

Antiserum production

Two female New Zealand white rabbits (approximately 6months) were immunized with PIBB protein by subcutaneously injecting at multiple sites on the back of the neck. The primary injection was given with 100 µg n (in 0.5 ml of PBS)mixed with 0.5 ml of FCA (Freund's Complete Adjuvant). Subsequent booster injections [100µg/dose) were given after mixing 0.5 ml of protein solution with 0.5 ml of FIA (Freund's Incomplete Adjuvant) on the day 21 and 28. The animals were blood 10 days after the last injection by puncturing the ear vein after giving local anesthesia. Serum was separatedfrom the blood, inactivated at

56^{0}C for 30 min, aliquoted and stored at -70^{0}C for subsequent use.

Evaluation of optimum antigen and antibody concentration needed for ELISA.

Purified PIBB protein and antiserum raised against it were subjected to checker boardtitration in ELISA to determine optimum antigen antibody titers using anti-rabbit goat antibody-horseradish peroxidase (HRP) enzyme conjugate and chromogen substrate as per standard procedure. Ninety-six well immunoplates were coated with different dilutions (0.25 to8. 0ug/ml) PIBB in duplicate wells (in 6 rows) in 50 mM carbonate-bicarbonate buffer (coating buffer) pH 9.5at 37^{0}C in an incubator, under humid condition for 3 hr. The plates were washed with PBS containing 0.05% Tween-20 (washing buffer/PBST), three times, with three minute intervals. The leftover sites were blocked with 100 µl/well 3% defatted milk powder in PBST (blocking solution) at 37^{0}C in an incubator for 1 hr. The wells were charged with serial doubling dilutions (50 to 1600) in blocking buffer of hyper immune serum with different dilutions (in 6 columns). The plates were incubated for 1hr at 37^{0}C and then washed thrice. After that plate was tapped to clear off any residual-washing buffer, the wells were loaded with 100ul /well of diluted anti-rabbit (whole molecule) goat antibody-HRP (SIGMA) conjugate diluted (1:40,000) in blocking buffer and incubated for 1 hr at 37^{0}C. The unbound conjugate was washed as described before and subsequently orthophenylenediaminedihydrochloride (OPD) solution (0.02%w/v in 0.01M acetate buffer containing 0.04% H_2O_2 (substrate solution)) was added to each well at the rate of 100µl/well and incubated. The reddish brown colour development was allowed to proceed for 20 min before it was stopped with 100µl/well of stopping solution consisting of 1M H_2SO_4. A_{492} of the colour development was measured in an ELISA reader. Antigen of 0.5 µg/ml and antiserum of 1:800 dilution was foundedoptimal(Fig 3 and 4)

Sensitivity and specificity of the ELISA for PIBB and antiserum against it.

Ninety-six well flat bottom- F96 MAXISORP NUNC-Immunoplates was coated with purified PIBB antigen (0.5µg/ml) at 4^{0}C overnight. The plates were washed with PBS containing 0.05% tween-20 (washing buffer/PBST) three times with three minute intervals as described before. The leftover sites were blocked with 100µl/well of blocking solution (PBST) at 37^{0}C in an incubator for a further 1 hr as described before. Antiserum at 1:400 dilution in blocking buffer was mixed with equal volume of purified PIBB at different concentration ranging from 1µg to 39 pg in PBST containing 3% Skimmed Milk Powder (SMP) for 1 hr at 37^{0}C in separate micro centrifuge tubes.

The PIBB, at different concentrations (19 pg to 1 µg /ml) was mixed with equal quantity of diluted antiserum (1:400 in PBST containing 3% SMP) was transformed to immunoplates coated with PIBB antigen (0.5µg/ml) and incubated at 37^{0}C for 1 hr. Following thrice washings antigen antibody reaction was detected using conjugate and substrate as described before. Percentage inhibition was plotted against dilution of the competitor (Fig. 5).

Cross reactivity of the antiserum raised against PIBB with E. coli expressed proteins was evaluated using expressed E. coli expressed GFP and E. coli and FMDV VP1-2A in indirect ELISA. In addition Anti-GFP antisera raised in rabbit and anti-FMDV VP1-2A rose in rabbit sera were also subjected to ELISA titration with PIBB as the coated antigen.

Standardization of Liquid Phase Blocking Enzyme Linked Immunosorbent Assay (LPBELISA)

Biomarker based competitive ELISA for detection of Sub-Clinical mastitiswas made with purified protein. Competitive ELISA was carried out as per standard protocols with modifications. Ninety-six well flat bottom (F96 MAXISORP NUNC) Immunoplates were coated with PIBB antigen at 0.5µg/ml in coating buffer at 4^{0}C overnight. The plates were

washed with PBSTthree times, with three minute intervals as described before. The leftover sites were blocked with 100µl/well of blocking solution at 37^0C in an incubator for 1 hr as described before. The defatted milk powder solution (3% in PBS) was spiked with PIBB protein (16µg/ml) was mixed with different dilutions (1:2 to 1:16 in PBS) with an equal volume of pre-diluted equal volume of pre-diluted (1:800 in blocking buffer) hyperimmuneserum and incubated for 1 hr at 37^0C. The serum-milk mixture was added in100 (l/well to the plate and incubated at 37^0C in an incubator for a further 1 hr. After washing the plates were tapped to clear off any residual-washing bufferand the wells were loaded with 100 µl/well, 1:40,000 anti-rabbit goat-HRP conjugate in blocking buffer
and incubated for 1 hr at 37^0C. The unbound conjugate was washed as described before and antigen –antibody reaction was detected by the addition of substrate (OPD- H_2O_2) solution as described before. The reddish brown color development was allowed to proceed for 15 minutes before it was stopped with100µl/well of stopping solution consisting of 1M H_2SO_4. A_{492} of the color development was measured in an ELISA reader.

Liquid phase blocking ELISA of milk samples collected from the field

Milk samples were centrifuged at 12,000 rpm for 5 min. at 4°C and supernant fat layer was discarded. A total of 200 µl of the clarified milk was added to a tube containing 200µl PBS and 400µl methanol. The mixture was then centrifuged at 10,000 rpm at 4°C for 5 min. An aliquot of 300µl of supernatant was subjected to detection. The results were expressed in percentage inhibition as follows:

$$\text{Percentage Inhibition (\%)} = (1 - A/A0) \times 100$$

Where, *A* is the absorbance of the well containing competitor and *A*0 is the absorbance of the well without a competitor.

RESULTS AND DISCUSSION

Cloning and expression

The purified protein up to 12 % SDS PAGE and CBB staining showed an intense single protein band of size 40kDa (Fig.2) which was absent in case of proteins of vector transformed cells. The size of the protein as per the derived amino acid composition is expected to be 20 kDa. Hence the observed 40 kDa may be a dimer of PIBB which was confirmed by affinity purification. The protein was over expressed when the cell grown in fermenter were induced with IPTG. A total of approximately 1.0 g of protein could be obtained from 1L culture. Fig. 3 and 4 show optimum concentrations of antigen and antiserum as detected by checker board titration. These are as 0.5 µg/ml for antigen and 1:800 dilution for antiserum under the standardized experimental conditions. Polyvalent Immunogenic Bovine Biomarker (PIBB) plasmid map is shown in (Fig.1).

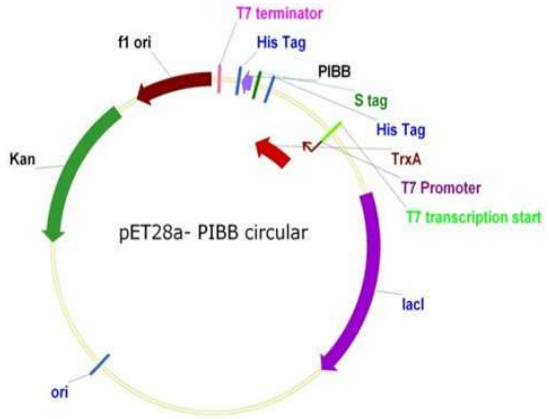

Figure. 1

Schematic representation of the pET28a-PIBB expression vector constructs. The vector constructs of 6239bp were made using pET28a vectors (In Vivogen) as a backbone.

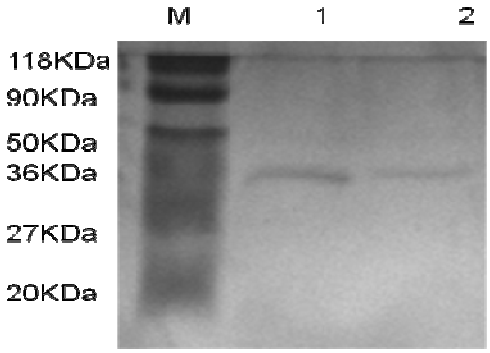

Figure. 2

***Column purified dimer protein (approx. 40KDa) from pET28a-PIBB transformed into E. coli BL21 (DE3) pLysS bySDS-PAGE analysis showed that the relative* molecular weight *of* His-tag *(4KDa) exists in the upstream of multi-clone site (MCS) of vector* pET28a.

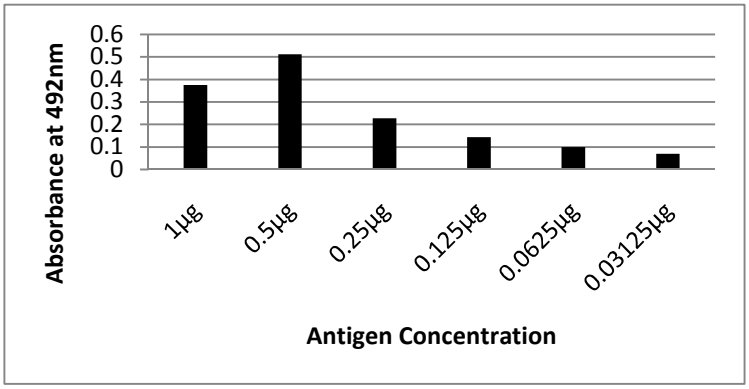

Figure.3

Antigen Concentration (0.5µg) a determination by checkerboard titration for blocking ELISA wells

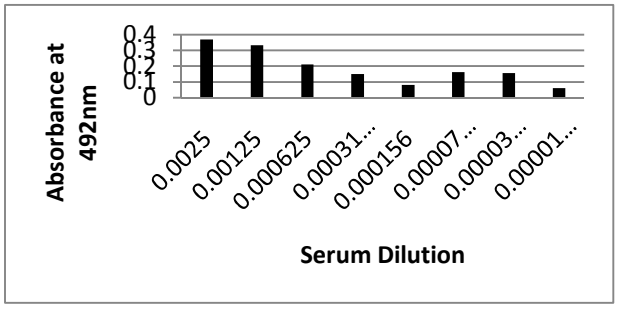

Figure.4
Antiserum titre optimization 1:800 for binding to biomarker in mastitis milk sample by checkerboard titration

Sensitivity and specificity of assay
The LOD, least detectable dose of PIBB was evaluated as the concentration of the antigen showing a 10% reduction in the maximum absorbance which is a 39pg / ml of PIBB diluted with 3% in blocking buffer (Fig. 5). As far as cross reactivity is concerned the expressed PIBB and the test antiserum against PIBB did not show any cross reactivity with antisera against various other expressed *E. coli* proteins available in the laboratory namely, anti-GFP and anti-FMDV VP1-2A protein antisera.

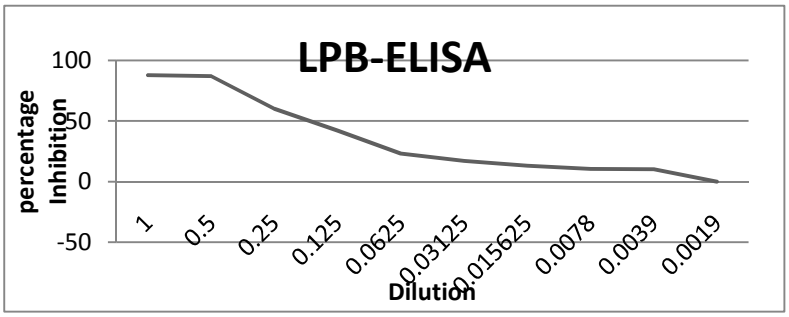

Figure.5
Determination of LOD of biomarker showing a 10% reduction in the maximum absorbance which is 39 pg/ml of PIBB diluted with 3% in blocking buffer

Development of blocking competitive ELISA
In this study, 87 mastitic milk samples were collected which has no history of treatment, 3 months before the experiment. As depicted in Fig. 6a of all the 87 milk samples 86 showed the inhibition of 30-50%. Fig. 6b clearly showed a proportionate correlation of percentage inhibition and SCC of milk samples. The cows studied were divided into 3 groups based on SCC, as high individuals with SCC of 15-20 lakhs/ml, mid individuals with SCC of 10-15 lakhs/ml and low individuals with SCC of 0.1-10lakhs/ml. The percent of positive samples detected was higher using biomarker, if inhibition of 30% by LPB ELISA was considered positive rather than using SCC cutoff values of > 5 lakhs/ml. There was a positive correlation between the % inhibition by LPB ELISA and SCC. As per our study, 86 of 87 (98.8%) cows showed positive for biomarker in milk which were subsequently diagnosed to show the presence of one or other pathogenic bacteria. On the other hand, one sample did not show any inhibition and the sample also showed low SCC (<5 lakhs/ml).

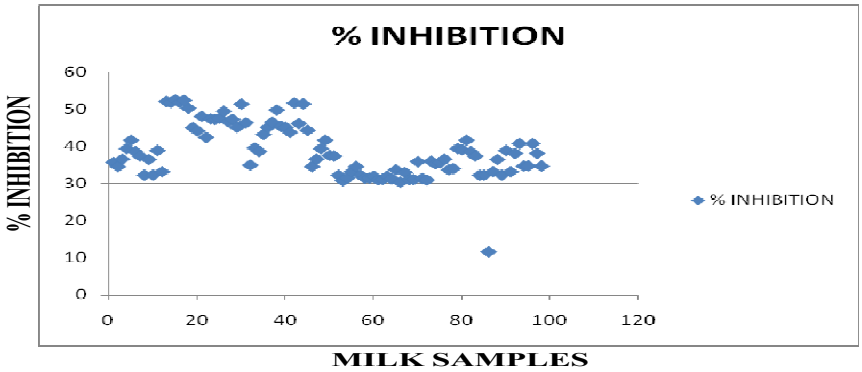

Figure. 6a

Percentage Inhibition in Liquid phase Blocking ELISA for detection on 87 m milk samples 86 showed the inhibition in the range of 30-50%

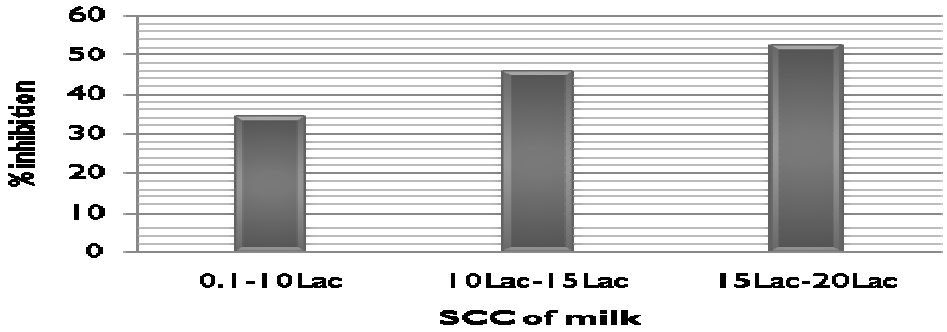

Figure.6b

Percentage Inhibition in Liquid phase Blocking ELISA for detection on 87 milk samples & Correlation with SCC into 3 different groups based on SCC, as high individuals with SCC of 15-20 lakhs/ml, mid individuals with SCC of 10-15 lakhs/ml and low individuals with SCC of 0.1-10lakhs/ml.

CONCLUSION

Mastitis in bovine is a major concern not only for the developed countries but also in the developing countries. The disease occurs mostly due to the poor hygienic conditions resulting in the infection of the udder through handlers and environment. Detection of mastitis at the subclinical level is of utmost important, as this helps in treating the disease with proper antibiotics. The present methods of EC and SCC are less reliable. Hence there is a need to develop highly specific penside test to regularly screen the milking cows to avoid any damage to udder due to infection that subsequently may lead to clinical mastitis. Biomarker based detection of the inflammations is highly reliable as the tissue shows quick response for any invasion. Thus detection of immune reactions in the udder through the detection of cytokines may be the method of choice. We have identified specific cytokines and developed LPB-ELISA for detection of bio-markers against mastitis. Initially the test may be used to undertake an interlaboratory comparison of the efficacy of biomarker based LPB-ELISA developed by us with the detection of mastitis by pathogen isolation and subsequently this may be adapted as a test of choice for routine screening.

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