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Bovine Mastitis: Novel Protein Treatment Strategy

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ABSTRACT

Mastitis is a major challenge to the worldwide dairy industry in spite of the widespread implementation of mastitis control strategies. The major economic loss of all forms of mastitis results from reduced milk production. Because of the difficulty in controlling mastitis the disease will maintain its role in the foreseeable future. Identifying genes offers the opportunity to improve production efficiency, quality through utilizing them in breeding programs, developing therapeutic agents that can be used to alter disease attributes by altering gene expression. The Lactoferrin gene having significant association with mastitis has been identified which is an iron binding protein present in biological fluids. This protein is synthesized by mammary epithelium cells and neutrophils and secreted as non-haem iron binding protein. It is a glycosylated protein having antibacterial, antiviral, immune-modulatory and iron haemostasis properties. In addition to it modulates the immune response by decreasing the free radical formation and by down regulating LPS induced cytokines and is an potent activator of immunological functions such as granulopoiesis, cytokine production, antibody synthesis, natural killer cell toxicity, lymphocyte proliferation and complement activation and production of interleukins (IL-1), (IL-2) and TNF. The lactoferrin acts as a carrier of IGFBP-3 and allows translocation of extracellular IGFBP-3 into nucleus of bovine mammary epithelium cells. Its concentration increases during dry period and during mastitis concentration may increases several folds. Moreover it modulates the immune response by decreasing the free radical formation and by down regulating LPS induced cytokines and exhibits strong

antimicrobial activity against a broad spectrum of bacteria (gram-positive & negative), fungi, yeasts and viruses and parasites. Lactoferrin acts as biomarker, antioxidant and vaccine adjuvant. This paper reviews the role of lactoferrin, its mechanism of action in regulation of mammalian host defense in combating mastitis which facilitates the inclusion of mastitis resistance in bovine breeding programmes.

Keywords: Lactoferrin, protein, biological fluids, immune system, antimicrobial, Mastitis

Mastitis is the most frequent and costly disease of dairy cattle. Losses due to mastitis can be attributed to both subclinical and clinical disease. Clinical mastitis losses are generally readily apparent and consist of discarded milk, transient reductions in milk yield and premature culling (Fetrow, 2000). Mastitis is a concern of most dairy farmers and their advisors due to these profound economic consequences. Mastitis-related losses are associated with reduction in yield, increased treatment costs, discarded milk, and increase in culling and associated dairy cow replacement rates. Lactoferrin is a component of natural protection system of animals and humans. It is 80 kDa bilobal glycoprotein, an iron binding protein predominantly present in biological fluids in milk, tears, saliva and in sweat (Park *et al.,* 2011) and also in specific granules of neutrophils that brings about adaptive and innate immune system (Legrand and Mazurier., 2010). Lactoferrin gene seems to be an important candidate gene for mastitis resistance trait (Seyfert *et al.*, 1996). Lactoferrin is present in the colostrum and normal milk of most mammals. Lactoferrin produced by mammary gland serves as a dual role, protecting the both mammary gland.

It was subsequently found to be an iron binding glycoprotein of the Transferrin family (Tf), discovered in 1939 as "red protein from milk" (Sorenson and Sorenson, 1939). The milk Lactoferrin is synthesized mainly by breast epithelium cells and neutrophils and secreted as non-haem iron binding protein (Plaffl *et. al.,* 2003). It is an glycosylated protein primarily having antibacterial, antiviral, immunomodulatory and iron haemostasis properties and in secondary granules of PMN,lactoferrin (Lf) is found in significant amounts. (15µg/10^6 neutrophils) (Gonzalez- Chavez *et. al.,* 2009). . The mean concentration of Lactoferrin content in bovines, goat and porcine ranges from 0.02- 0.2 mg /ml. Lactoferrin content in human milk is 4.2+ 1.2mg/ml and in horse and mouse is 0.2- 2 mg/ml and sheep

20-200ug/ml (Shimazaki *et al*, 2000). Lactoferrin concentration increases during the dry period and during mastitis incidence in bovines and their concentration may increase by about 100 folds during bacterial diseases. The different concentration of lactoferrin in different stages is regulated by lactoferrin gene has been reported. (Guo Hua *et al.,* 2004).

Structure and properties of lactoferrin

Lactoferrin consists of single chain protein of 708 amino acids with 19 amino acid signal peptides (Pierce *et al.,* 1991). The protein is glycosylated and position of potential glycosylation sites may vary among proteins. The amino acid sequence of Lactoferrin has N-glycosylation sites as Asn-233, Asn-281, Asn-368, Asn -476 and Asn-545 . Glycosylation of Lf affects the ability to bind iron (Baker and Baker, 2009). Lf is capable of binding Fe^{2+} or Fe^{3+} ions and can bound to Cu^{2+}, Zn^{2+} and Mn $^{2+}$ ions. The coding sequence of the functional bovine Lactoferrin protein defines a single polypeptide chain of 708 amino acids, which is folded into two separate lobes (N and C terminals) that each contains two-iron binding and two bicarbonate-binding sites.

The promoter region consists of 1122 bp and has a non-canonical TATA box at 25 bp with in GC rich region that can bind to sp-1 and sp-3 factors (Zheng *et. al.,* 2005). The three dimensional structure of lactoferrin was identified in cow (Moore *et al*., 1997), buffalo (Karthikeyan *et al*., 1999), camel (Khan *et*. *al.,* 2001) and horse (Sharma *et al.,* 1998) showed that this protein among different species had 90% sequence homology and had similar iron binding sites. The lactoferrin coding regions of about 60 different species analysed had variable length of gene (2055-2190) residues, due to deletions, insertions and mutations in the stop codon. Lactoferrin is expressed both constitutively and constituely on mucosal surfaces while in some tissues it is induced by external agents.

Lactoferrin (Lf) is basic, positively charged protein with an isoelectric point of 8.4- 9.0 which is important to iron activity and ability to bind different cell types and anionic macromolecules (Farnaud and Evans, 2003). It exerts both bacteristatic and the bacteriocidal activity is by binding to iron and bacteriocidal is by interaction of lactoferrin and bacterium.

Growth and development of the gastro- intestinal tract in new born animals fed their dams milk are known to be more rapid than in those fed

commercial formulae. Lactation alters microbial population in the gut of non- ruminants and increasing pre-weaning weight gain (Joshi *et al.,* 2002; Roblee *et al.,* 2003). Lactoferrin is present in the colostrum and normal milk of most mammals. The interaction of Lactoferrin with proteins such as immunoglobulin G or casein, in non-lactating mammary secretion may affect the anti-microbial properties of lactoferrin. The amount of Lactoferrin synthesized in mammary gland is controlled by prolactin, whereas in reproductive tissue it is determined by estrogens (Teng *et al.*, 2002). The biological properties are mediated by specific receptors on the surface of the target cells. These receptors are present on each cell type. Lactoferrin is metabolized either through receptor mediated endocytosis of the phagocytic cells or through the direct uptake by liver.

The protein is present in variable amounts in humans, mouse, mare and sow milk but rat, rabbit and dog appears to be devoid of lactoferrin.

Lactating and non-lactating healthy cows

Lf produced by mammary gland serve as a dual role, protecting the both mammary gland and neonatal intestine from infection. Growth and development of the gastro-intestinal tract in new born animals fed their dams milk are known to be more rapid than in those fed commercial formulae (Heird *et al.,* 1984). The interaction of Lactoferrin with proteins such as immunoglobulinG or casein, in non-lactating mammary secretion may affect the anti-microbial properties of lactoferrin. The fully involuted udder is resistant to coliform infections, mostly due to high content of Lactoferrin. The protein concentrations have been reported to increase, over 100 fold those in normal milk, and throughout the non-lactating period Lf concentrations remain high. The dramatic increase in Lactoferrin concentrations after cessations of milking appears to be a manifestation of process of involution, while during normal lactation Lf is a minor milk protein (Masson and Hereman, 1971).

Concentration of lactoferrin in secretions of the bovine mammary gland in healthy and mastitic cows

Lactation stage	Lf conc. mg/ml	Reference
Immediately after parturition	2.4	Nonnecke & Smith. (1984)
Lactating cows	0.02-0.35	Welty *et al.* (1976)

2-4 days of involution	2.4	Welty *et al.* (1976)
5-7 days of involution	2.6-17.8	Welty *et al.* (1976)
Sub-clinical mastitis	0.2-1.2	Hagiwara *et al.* (2003)
Clinical mastitis	0.3-2.3	Kawai *et al.* (1999)

Lactoferrin concentration in milk from quarters infected with *Staph. aureus* or Streptococci were significantly higher than in milk from quarters infected with coagulase negative *Staphylococcus* and *Corynebacterium bovis* (Hagiwara *et al.,* 2003). Lactoferrin concentration in milk ranges from 0.3-2.3mg/ml, these concentrations are generally higher in normal cows or those with sub-clinical mastitis (Kawai *et al.,* 1999; Hagiwara *et al.,* 2003). Mutual interaction among the vitamin-A, IGFBP-3 and Lactoferrin modulates the proliferation and apoptosis of mammary epithelium. (*Puvogel et al.,* 2005). During mastitis bicarbonate leaks from the blood due to increased permeability. Lactoferrin sequesters free ferric ions present in milk (Sordillo and Streicher, 2002). The bilobal protein has been known to exist in more than one isoforms. Furmanski *et al.* (1989) reported the RNase Lf (an isoform of human milk having antigenic,chemical and physical properties but differing in possession of potent nuclease activity and lack of significant iron binding capacity).

Functions of lactoferrin

Many studies have examined the effects of bovine lactoferrin on the immune system. It regulates the inflammatory responses by binding to bacterial endotoxin, which inhibits formation of proinflammatory cytokines.. Lactoferrin appears to bind uniquely to the region of MHC proteins and CD_4 as well CD_8 determinants on T_4 (helper) and T_8 (Supressor) lymphocytes, it bears sequence homology with MHC- class II determinants. It has immunoregulatory function that affect the antibody synthesis and complement activation.

Lactoferrin modulates the immune response by decreasing the free radical formation and by down regulating LPS induced cytokines (Haversen *et al*, 2002). The protein inhibits the biofilm formation (Singh *et al*., 2002). Ingestion of bovine in particular, it has been evident that oral bovine lactoferrin (bLf) has beneficial effect on digestive and non–digestive tract tissue in various animal models. Bovine lactoferrin administration reduced

the lung consolidation score and the number of infiltrating leukocytes in brochoalveolar lavage fluids (Yamauchi, K. *et. al.,* 2006).

Oral bovine Lactoferrin activates the transcription of immune related genes in the intestine, and such transcriptional activation may promote systemic host immunity. Intake of bovine Lactoferrin increases the expression of IL-12p40, IFN-beta and NOD2. Consequently Lactoferrin f can exists in association with Fe^{3+} (holo-lactoferrin), or Lf can exist free of Fe^{3+} with different three dimensional conformations (Baker and Baker *et al.,* 2004). Apo lactoferrin has an open conformation while the iron bound holo- form of Lf is an closed molecule.

Treatment of Mastitis with lactoferrin

Bovine lactoferrin showed marked bacteriostatic activity in vitro against against mastitic bacteria, Ecoli. The first helix of Lf forms the major part of the bacteriocidal domain as Lactoferricin domain (Bellamy *et al.,* 1992). The bactericidal domain can serve as macromolecule binding site. The positive charge at the N terminal residues and the adjacent C terminal end of the first helix form the binding sites for bacterial LPS. A second putative antimicrobial domain, Lactoferrampin is present in the N-1 domain of the bLf (Vander kraan *et al.,* 2004). Lactoferrin alone or in combination with antimicrobial drugs in the

treatment of bovine mastitis are used . Lactoferrin with its broad-spectrum antimicrobial effect would be a good candidate for a non-antibiotic treatment of infections (Masson et al. 1966). Lactoferrin infusion could potentially be useful in the treatment of bovine mastitis, and could partly replace the use of antimicrobials. Another application could be the prevention of mastitis at drying off (Kai *et al.* 2002b) and endogenous lactoferrin infusion of during this time. Clinical mastitis is, however, rare in the middle of the nonlactating period (Nonnecke & Smith 1984a), when the concentration of Lactoferrin in mammary gland secretion is at its highest (Welty *et al.* 1976). Kai and co-workers (2002b) suggested the same as speculated here: administration of exogenous Lf during early involution could help limit bacterial growth, not only for its bacteriostatic and bactericidal effect, but also for the priming effects on the innate immunity of the host. In addition, a high concentration of Lf promotes phagocytic activity in the mammary gland and the activation of bovine complement (Kai *et al.* 2002a). Lf could

affect the disposition of apoptotic cells and bacteria during the early non-lactating period, and exogenous Lf could thus be used as a dry-cow therapy.

The early period of mammary involution coincides with a period of increased susceptibility to intramammary infection, Its antimicrobial action differs from that of Lactoferricin but physiologically the localization of the peptides in the N -1 domain allows the protein to bind to the membrane surface and intiates the bacteriocidal activity by disturbing the membrane integrity. The lactoferrin is regulated by the type of cell producing the protein. The biological properties are mediated by specific receptors on the surface of the target cells. These receptors are present on each cell type.

The beneficial effects of Lactoferrin to exhibit strong antimicrobial activity against a broad spectrum of bacteria (gram-positive & negative), fungi, yeasts, viruses and parasites and has beneficial effects on lactobacillus and bifidobacterium (Garcia *et al*., 2011). Antimicrobial activity of lactoferrin affecting the bacterial cell wall occurs due antimicrobial peptides of an N-terminal part aminoacid chain of this protein, lactoferricin and lactoferrampin. These proteins are released from native proteins by pepsin mediated digestion. It represents one of the first defense systems against microbial agents invading the organism mostly via mucosal tissue.

Evidence has been presented that the biosynthesis of lactoferrin can increase during certain bacterial infections, and these discoveries provided further evidence that support the idea of an important role in host defense. Consequently, it is now widely accepted that lactoferrin is an important part of our innate immune system (Legrand and Mazurier 2010; Valenti and Antonini 2005; Ward *et al.* 2005). It has bacteriosatatic action by binding to free iron which is essential for the growth of bacteria. A lack of iron inhibits the growth of iron-dependent bacteria such as *E. coli*. Bactericidal activity is mediated by affecting Gram-positive bacteria is mediated by electrostatic interactions between the negatively charged lipid layer and the positively charged lactoferrin surface that cause changes in the permeability of the membrane (Valenti and Antonini, 2005). Lactoferricin, a cationic peptide generated by the pepsin digestion of lactoferrin, has more potent bactericidal activity than the native protein. There are two forms known at present: lactoferricin H (derived from human lactoferrin) and lactoferricin B (of bovine origin) (Bellamy *et al.,* 1992).

Lactoferrin is an potent activator of immunological functions such as granulopoiesis, cytokine production, antibody synthesis, natural killer cell toxicity, lymphocyte proliferation and complement activation and production of interleukins (IL-1), (IL-2) and tumor necrosis factor (TNF). (Kimber *et. al.*, 2002).

The lactoferrin dependent cytokine mediated stimulation of activity of NK cells and lymphocytes stimulation CD- 4+ and CD-8+ plays important role in defence against tumor cells. The parasiticidal role of Lactoferrin has been identified as it acts against parasites, the infectivity of toxoplasma-gondii, eimeria stiedai and Sporozoites are reduced after their incubation with lactoferricin-b.I t breaches parasitic membrane integrity and causes subsequent interactions between host and parasite (Omata *et al.*, 2001). The beneficial effects of Lactoferrin has a positive effect on wound healing. Studies with various animal models indicate that recombinant human lactoferrin can stimulate the closure of wounds in vivo (Tang *et al.* 2010b). Oral lactoferrin have direct effects on diseases of the colon, such as inflammatory bowel disease or diarrhea (Ha and Kornbluth 2010; Ochoa *et al.* 2012). Studies have found that human lactoferrin modulates the intestinal flora in piglets (Hu *et al.* 2012).

CONCLUSION

Lactoferrin is a prominent component of the defense system. Due to its unique antimicrobial, immunomodulatory and anticarcinogenic properties, lactoferrin contributes to mammalian host defense. Bovine mastitis remains a complex disease and its management is an increasing challenge. The progress has been made to a great extent by the use of therapeutic and prophylactic antibiotics treatments. But there is resistance problem against many antibiotics due to concurrent change in the etiology of bovine mastitis. Similarly, there is also the problem of the efficacy and cost-effectiveness for the prophylactic application of intramammary antibiotic.

In future, these challenges provide an opportunity to researcher and clinician to study the genetic aspects of the disease and reduce antibiotic usage, developing fast and reliable diagnostic techniques with cost-economy.. Given the current interest and the subsequent clinical trials with lactoferrin, this multifactorial protein may become useful in combating

mastitis which facilitates the inclusion of mastitis resistance in Animal breeding programmes.

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