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Bovine Immunogenetic Response to *S. aureus* and *E. coli* Mastitis: A Review

Jyotishree Bayan and Sourabh Sulabh

Department of Animal Genetics and Breeding, College of Veterinary Science and Animal Husbandry, Assam Agricultural University, Khanapara, Guwahati, India

Corresponding Author: sourabhjsr.vtro4@gmail.com

Abstract

The difference in the pattern of immune response between *Escherichia coli* and *Staphylococcus aureus* leads to different form of mastitis that they cause. A more hyper immune response by *E. coli* results in clinical mastitis whereas a subdued response of *S. aureus* changes the outcome to subclinical mastitis. The immune system of the animal recognises various antigens present in these bacteria (such as lipopolysaccharide, peptidoglycan and lipoteichoic acid) which induce or suppress the activity of major immune related genes resulting into the type of outcome the animal shows which either help the animal to recover quickly or may even amplify the response in such a way that causes discomfort or may even lead to death of the animal. This review reflects the importance and role of immune related genes involved in the mechanism of protection of the animal against two of the most common infectious bacterial agents, i.e., *E. coli* and *S. aureus* responsible for inducing inflammatory response in the mammary gland.

Key words: Bovine mastitis, Molecular markers, *Staphylococcus aureus*, *Escherichia coli.*

Introduction

Bovine mastitis is defined as an inflammation of the mammary gland in response to injuries or invasion of the mammary tissue by foreign germs like bacteria, fungi, viruses etc. The inflammation thus produced serves to destroy and neutralize infectious agents along with encouragement of healing process so that normal function can be renewed (National Mastitis Council, 1996). Based on traditional cost analysis and data survey mastitis has been identified as economically most important disease in dairy cattle (Wells *et al*., 1998). Bangar *et al.* (2014) conducted a meta-analysis by pooling data obtained from 28 studies in India, and found the prevalence of subclinical mastitis to be 46.6%. Species of the pathogen and associated virulence factors are one of the most important elements influencing the results of intramammary infections (IMI) (Harmon, 1994; Pearson and Mackie, 1979). A large number of microorganisms (more than 137), which includes bacteria, yeast, fungi and algae are responsible for causing bovine mastitis (Watts, 1988). However, only five species of bacteria (*Staphylococcus aureus*, *Streptococcus agalactiae*, *Strep. dysgalactiae*, *Strep. uberis* and *Escherichia coli)* account for most of the infections leading to bovine mastitis in majority of the countries including India (Hedge *et al.,* 2012). The most common causative agent for causing mastitis may differ according to geographical distribution, as the prevalence of mastitis-causing bacteria display geographic variation (Olde Riekerink *et al.,* 2008). Immune response controlled by

regulation of genes plays a crucial role in tackling this disease. Improved immune response can be brought by selection for disease resistance in livestock, which is considered as the most important prophylactic approach for improving the animal wellbeing (Mallard and Wilkie, 1999; Kelm *et al.,* 2001; Stear *et al.,* 2001). Thus, the incidence of disease can be reduced through improved genetic potential for enhancement of animal welfare, health and food quality, as well as decrease in production costs. One of the most important methods to increase the efficiency of selection in dairy cattle for increased resistance is to identify specific genes or genetic combinations related with complex infectious disease such as mastitis (Tao and Mallard, 2007).

Pathogen specific response for eliciting inflammation

Escherichia coli is a Gram-negative coliform bacteria and its invasion of bovine udder causes heavy, acute inflammatory response in the mammary gland leading to severe clinical symptoms. Whereas, infection caused by other bacterial microbes, such as Gram-positive *Staphylococcus aureus* or *Streptococcus uberis*, usually results in mild or less severe clinical signs and symptoms producing subclinical mastitis (Bannerman *et al.,* 2004; Smith and Hogan, 1993). Quick and effective recognition of these pathogens by host immune system is of crucial importance in showing a rapid innate immune response acting as a key outcome determinant to the infection (Bannerman, 2009). Mastitis associated immune response is a very complex biological process, activating not only recruited and resident immune cells, but also involves mammary epithelial and endothelial cells. Both acute as well as chronic mastitis give rise to substantial increase in somatic cell count (SCC) in milk (Paape *et al.,* 1979; Burton and Erskine, 2003; Rainard and Riollet, 2003). Characteristic dissimilarity in the innate immune response, measured as observed differences of soluble inflammatory mediators in milk has been reported after injection of the Gram-positive and Gram negative bacteria into the mammary gland (Bannerman *et al.,* 2004). Cellular defence of the milk and soluble effector molecules, such as cytokines, complement factors and acute phase proteins (APP), signifies important portions of the innate immunity in the mammary gland. Importance of Cytokines during the course of intra-mammary infection (IMI) and the way of expression in milk that are evoked by various mastitis pathogens have been well distinguished (Bannerman, 2009). Pro-inflammatory cytokines such as interleukin *IL-1*β*, IL-8, IL-12,* interferon *IFN-*γ and tumor necrosis factor *TNF-*α increased concentration in milk during IMI has been observed. Gram-negative bacteria like *E. coli* have produced a greater response than Gram-positive bacteria or mycoplasma in milk obtained from infected quarters (Bannerman *et al.,* 2004; Kauf *et al.,* 2007). Inflammatory response control is guided by up-regulation of anti-inflammatory cytokine, such as *IL-10* and *IL-4* to counteract the effects of excessive release of pro-inflammatory cytokine, such as *TNF-*α and *IFN-*γ during IMI caused by several bacteria which are able to induce a suitable immune response. Whereas, it was observed that *S. aureus* did not induce sufficient response in the host (Bannerman *et al.,* 2004). Though some reports suggest the ability of *S. aureus* to induce inflammatory response of various cytokines but the extent of response was comparatively less than *E. coli* (Strandberg *et al.,* 2005). However, levels of most cytokines declined after 4 to 6 h of stimulation of culture cells.

Acute phase response associated molecules characterized an early defence mechanism against infection. Haptoglobin (HP), serum amyloid A (SAA) and lipopolysaccharide binding protein (LBP) are some of the most important molecules in milk, produced during an IMI brought about by injecting *E. coli, S. aureus, or S. uberis* into the bovine udder (Bannerman *et al.,* 2004). Milk somatic cells (MSC) obtained from quarters found positive for either *E. coli* or *S. aureus* showed higher levels of pro-inflammatory cytokines mRNA, like *IL-6, IL-8, IL-12*, *GMCSF* and *TNF-*α in comparison to those collected from control quarters; with more magnitude and rapidity of gene expression was found to be strong during *E. coli* than *S. aureus* mastitis (Lee *et al.,* 2006). Milk somatic cells (MSC) from *S. aureus* infected cows, produce proinflammatory cytokines, chemokines and their receptors were found to be significantly activated (Tao and Mallard, 2007). Chemokines such as *RANTES* and *CCR8*; cytokines like *M-CSF* and *IL-17* and antigen presenting molecules, such as *TAP2* and *MHC1*, as well as pattern recognition molecules, *ICAM3* and *CD14* were observed to be up-

regulated in somatic cells obtained from milk of infected quarters in comparison to control. These results towards the condition after recognition of invading microbes, MSC start a signal to recruit factors comprising of cellular components of the immune defence from the blood to the mammary gland. This is in accordance with the findings of Alluwaimi *et al.* (2003), who reported that MSC obtained from quarters affected with *S. aureus* showed a pronounced increase in mRNA expression for several cytokines *(IL-6, IL-12, TNF-*α and *GM-CSF)* involved in the activation of the inflammatory response. However, in the same study the levels of *IFN-*γ and *IL-2* mRNA continuously decreased as the infection proceeded and developed further. *S. aureus* has the ability to suppress these two cytokines, which may be related to the reported potential of this pathogen to inflict a shift in the population of T cells from cells expressing CD4+ to those expressing CD8+ markers (Park *et al.,* 1993; Riollet *et al.,* 2000).

Mastitis caused by *Staphylococcus aureus*

Staphylococcus aureus usually produces chronic and subclinical forms of mastitis and is one of the most common microbes that cause mastitis. *S. aureus* produce various toxins in the mammary tissue and the body that destruct cell membranes and can also damage tissue involved in production of the milk. Leukocytes get attracted to the region of inflammation, where they endeavour to quarrel out the infection. Fresh entry of bacteria causes damage of tissues that surrounds the teats and the gland cisterns within each quarter, which finally leads to formation of scar tissue. The bacteria after entering the mammary gland move up the duct system and accommodates itself in deep-seated pockets of infection in the alveoli (milk secreting cells), followed by the development of abscesses that cut off the bacteria and prevents its spread but it results into bacteria able to avoid detection by the immune system. Thus, abscesses so formed prevent antibiotics from reaching the bacteria and are the most important cause for the poor treatment to response (Petersson-Wolfe *et al.,* 2010). Bacteria have the tendency to resist the killing effects of certain antibiotics by hiding inside neutrophils and other host cells. Neutrophils attempt to throw out the bacteria, but many of them survive and become dormant within them, resulting in prevention of contact with antibiotics. Finally, when the leukocytes die (usually within two days) the bacteria are again released to restore the infection process (Petersson-Wolfe *et al*., 2010). At the time of infection, destruction of alveolar and ductal cells reduces yield of milk. These vandalized cells merge with white blood cells and cause blockage of the milk ducts that drain the alveolar mass, contributing to more scar tissue formation and reduction in production of milk. At a later time the ducts may again open, but this usually follows the release of *S. aureus* bacteria to surrounding areas of the udder. The invasion of *S. aureus* to other areas within the gland leads to the formation of new abscesses that has the tendency to become quite large and is detected as chunks within the udder (Petersson-Wolfe *et al*., 2010).

Tao and Mallard (2007) screened Holstein cows suffering from *Staphylococcus aureus* mastitis. They isolated blood mononuclear cells and milk somatic cells from these animals to investigate the genes involved in immune system response through micro-array analysis and found a total of 22 genes in blood mononuclear cells and 16 genes in milk somatic cells to be differentially expressed. Genes such as *IL-8* and *IL-18* were found to be significantly up-regulated. Stimulation of epithelial cells of mammary gland with *Staphylococcus aureus* culture supernatant, *TLR-2* receptor was found to be activated and also found to be associated with *AP-1* and *IL-17A* signalling pathways (Gilbert *et al.,* 2013).

ARK-Genomics bovine cDNA array was used and identification of differentially expressed genes was carried out after infecting monocyte-derived macrophages with *Staphylococcus aureus in vitro.* A total of 420 genes were found to be differentially regulated in which *Interleukin 4* and *Interleukin 13* were identified as putative expression quantitative trait loci. *TNF* superfamily member 5 (*CD40* legend) was identified as an upstream regulator (Lewandowska-Sabat *et al.,* 2013). Primary epithelial cell cultures isolated from milk were treated with heat inactivated gram-negative *E. coli,* gram-positive *S. aureus* and *Streptococcus uberis.* Treatment for 6 h with *E. coli* and *S. aureus*

significantly enhanced mRNA expression of *IL-8, IL-6,* serum amyloid A (*SAA*) and *TNF-α*. *Streptococcus uberis* in the same concentration was able to only induce the expression of *IL-8* after a 6 h of treatment (Wellnitz *et al.,* 2006).

Peptidoglycan (PGN) and lipoteichoic acid (LTA) forms an important component of Gram positive bacterial cell wall like *S. aureus.* These two complex molecules also act as important inducer of inflammation when bacteria invade mammary tissue. Peptidoglycan support is formed of carbohydrates which include alternating units of N-acetylglucosamine and N-acetylmuramic acid. The N-acetylmuramic acid residues are cross-linked to peptides. Rigidity and support to the cell wall of the Gram positive bacteria is provided by peptidoglycan. They are made up of several layers of peptidoglycan, providing noteworthy mechanical strength. The key immune recognition is based on common structures of the invading microbes. Bacterial cell component such as peptidoglycans, peptidoglycan recognition protein (PGRP) and Lipopolysaccharides induces immune reactions which includes release of cytokines to reaction of the body in the form of increase in temperature resulting in fever (Doyle and Dziarski, 2001). Peptidoglycan and lipoteichoic acid present in the cell wall of gram-positive bacteria, one of which is *Staphylococcus aureus* have been recognised as a potent antigen having ability to stimulate the immune system of the host (Tilahun *et. al.,* 2014). Peptidoglycans act via activating the Toll-like receptor 2 (*TLR-2*) and can be used for the stimulation of lymphocytes and monocytes (Matsui and Nishikawa, 2012).

The structure of Lipoteichoic acid (LTA) is made up of long chains of glycerol phosphate or ribitol. Lipoteichoic acid (LTA) is attached to the cell membrane with the help of a diacylglycerol molecule. LTA is a teichoic acid which remains attached to a lipid present on Gram-positive bacterial cell wall. LTA is one of the pathogen-associated molecular-patterns (PAMP) and is recognized by Toll-like receptor 2 (*TLR-2*), finally leading to the activation of *NF-kB*. LTA was used to elicit immune defence response in bovine mammary epithelial cells by *in vitro* challenge. A significant increase in mRNA expression for *IL-1β, IL-8, TNFá, CXCL6* and *â-defensin* was noted during the study (Strandberg *et al.,* 2005). In a study using PGN and LTA for stimulating peripheral blood mononuclear cells it was found that *TLR-2* acts as major receptor influencing the outcome of cytokine response in which several cytokines (*IFN-γ, TNF-α, IL-8 and IL*-10) were found to be up-regulated (Sulabh *et al*., 2016).

Mastitis Caused by *E.coli*

E. coli mastitis (Coliform mastitis) most commonly leads to a final outcome of quick elimination of bacteria or sometimes prolonged infection or even death of the animal due to endotoxin shock which describes the potential of the cow to limit harmful inflammatory reactions and to get rid of the infection (Burvenich *et al.,* 2003). Mastitis caused by *E. coli* has a typical sudden onset, which results in peculiar changes in the appearance of the milk, initially to yellow and serous and later to thick and clotty. High increase in somatic cell count (SCC) of the milk is observed. The mammary gland becomes either tender or hard and swollen. The cow also shows systemic signs, including high fever, increase in frequency of the pulse, reduced contractions of rumen, loss of appetite, decreased and depressed milk production. Experimental *E. coli* mastitis models have indicated that the initial signs are most commonly noticed at the local level; with about 8 h post challenge, whereas fever and other systemic signs peak at 12 h post-challenge (Hirvonen *et al.,* 1999; Haddad *et al.,* 2001). During mild to moderate infection, the systemic signs disappear within 48 h followed by vanishing of local signs within 7 days (Hirvonen *et al.,* 1999). During severe cases, the animal may not survive and the systemic signs may remain extended resulting in permanent loss of milk production (Rantala *et al.,* 2002). Dego *et al.* (2012) studied expression profiles of genes in primary bovine mammary epithelial cells co-cultured with strains of *E. coli* associated with acute or persistent intra-mammary expression (IMI). They used micro-array to obtain and analyse the data which revealed a common set of genes to be differentially regulated during both acute and persistent IMI.

Lipopolysaccharide (LPS) acts as the most important inflammatory inducer for Gram negative bacteria. The large molecule made up of polysaccharides are composed of O-antigen and a lipid

in which the outer and inner core are joined together by a covalent bond. LPS are part of the outer membrane of Gram-negative and have the potential to generate powerful responses of the immune defence. LPS attaches to *TLR-4/CD14/MD2* receptor complex and initiate cytokine release in various cell types (Gerold *et al*., 2007; Lu *et al*., 2008). LPS play an important role in activating many transcription factors (Reid *et al*., 1997).

Lipopolysaccharide is a vital constituent of the outer membrane of Gram-negative bacteria such as *E. coli* and contributes immensely to the bacterial structural integrity. LPS also acts as an endotoxin, which induces an aggressive response from animal immune system. The innate immunity is the first line of host defense in mammals and is involved in detecting a wide variety of invasive pathogens. The receptors of the innate immune system are initiated by antigenic parts of pathogens; one of which is cell membrane component of Gram-negative bacteria such as LPS, involved in the activation of the immune response (Schnare *et al.* 2001). Thus, LPS forms a major marker for the host's recognition when Gram-negative bacteria are the intruding pathogen. LPS interacts with cells leading to the establishment and release of a large gamut of inflammatory mediators involving cytokines and chemokines that are of utmost importance for initial innate and later adaptive immunity acting against the bacterial defense (Beutler *et al.,* 2003). Under usual circumstances, a well managed cellular response to bacterial antigens gives protection to the host from infection. But hyperactivation of the immune response, results in excess synthesis and release of various proinflammatory cytokines in huge amount leading to cellular injuries and tissue damage (Pinsky, 2004). Several anti-inflammatory cytokines like *IL-10* are released from cells in order to regulate and control the over expression and release of pro-inflammatory cytokine like *IFN-*γ and more important *TNF-*α*.* But their late release or response may severely affect the health condition of the suffering animal.

Expression profiling of immune related genes by real time qRT-PCR was studied by i*n vitro* cell culture model of bovine monocyte derived macrophage after exposing it to lipopolysaccharides derived from *E. coli* (Taraktsoglou *et al.,* 2011). LPS caused significant upregulation of *TLR-4* via a *MYD88* independent pathway, which resulted in significant upregulation of genes involved with *NF-kB* signalling pathway accompanied by the expression of pro-inflammatory cytokine (*TNF, IL-*1β*, IL-6*) and chemokine genes (*IL-8, CCL-5, CCL-3*). Micro-array analysis of immune response of pigs following stimulation of porcine PBMC with lipopolysaccharides revealed a general inflammation response with over-expression of *SAA1*, pro-inflammatory chemokines *IL-8, CCL-2, CXCL-5, CXCL-3* and *CXCL-2* as well as genes related to oxidative processes and calcium pathways (Gao *et al.,* 2010). A marked increase in mRNA expression for *IL-*1β*, IL-8, TNF-*α*, CXCL-6* and *â-defensin* was observed when LPS was used to challenge bovine mammary epithelial cells (Strandberg *et al.,* 2005).

Conclusion

Study relating to response of the immune system during invasion of several types of bacteria helps in understanding the mechanism and way of control of the action of various cytokines. Excessive release of pro-inflammatory cytokines during *E. coli* infection causes tissue injury, fever and even death during the acute inflammation. This can be effectively controlled by injecting proper dose of anti-inflammatory cytokines which regulate the uncontrollable release of pro-inflammatory cytokines. On the other hand chronic mastitis caused due to *S. aureus* invasion of the mammary tissue is due to ineffective release of pro-inflammatory cytokines. So in this case efficiency of the immune system can be increased by injecting proper amount of pro-inflammatory cytokines so that a more aggressive response of the body leukocytes to the site of inflammation can be generated at an appropriate time. Overall the way in which the complex system of cytokines in combination with inflammatory cells, influences the outcome of the disease such as mastitis to a great extent. Analysis of the network helps us in guiding to a better understanding and finally to a better way of treatment and control of the disease.

Conflict of interest: Authors declare no conflict of interests that could possibly arise.

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