



## Importance of Bovine Mastitis Associated Gene Expression Analysis – A Review

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### ABSTRACT

Bovine mastitis has for a long period severely restrained production performance in the dairy enterprise. Despite improved management approaches and veterinary services, mastitis is still responsible for a major loss of the economy to the extent of worldwide. Genetic control as in development of protective immune mechanism and disease tolerant animal is considered an economic and prophylactic technique for health improvement management. We, therefore, need to recognise thoroughly the factors regulating the association between both the etiological agents and the host's mammary gland cells. Consequently, we need to determine differentially expressed genes during particular conditions in regard to mastitis. High-throughput transcriptome sequencing technique (RNA-Seq) has evolved as the main option for the analysis of differential gene expression setting the foundations for modern genetic research.

### HIGHLIGHTS

- Mastitis in bovine is among the highly prevalent and devastating disease condition globally.
- RNA-Seq is a preferred tool to map and quantify transcriptome profile.
- To unfold the underlying functional elements of the genome entirely and their inter-relationship with the mastitis.

**Keywords:** Mastitis, Disease resistance, RNA sequencing, Gene expression

World milk production has steadily increased gradually with time. Global milk production achieved 852 million tonnes in 2019, an expansion of 1.4 percent against 2018, mainly due to increases in production from key producing regions like Asia. Milk production in Asia rose by 10 million tonnes to almost 360 million tonnes, or 2.9 percent against 2018, across over 90 percent coming from India and Pakistan (FAO, 2020). Besides the rising demand from fast growing urbanization, milk productivity is limited in several developing countries due to poor-quality feed services, diseases, limited market, and service access and the low genetic potential of milk producing dairy animals. Therefore, the rise in the supply to meet the demand should be viewed with caution as there are negative genetic associations between milk production and both fertility as well as development of diseases, suggesting that genetic depletion of fertility and health results mainly

from selection for increased milk yield (Kumar *et al.*, 2017) indicative of the mastitis effect is not only limited to the udder. Despite improvements in management practice and veterinary care, mastitis has also risen in many countries over the years. Genetic immune response regulation and disease resistant selection in livestock were very well known and viewed as an economical and standard treatment solution to improving animal health status. And to significantly improve dairy cattle selection with increased resistance, we need to first understand the associated gene (Buitenhuis *et al.*, 2011; Kemper and Goddard, 2012; Goddard *et al.*, 2016) and their expression profile in reaction to the pathogen-related to mastitis via transcriptome profiling.

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## BOVINE MASTITIS

The disease of great economic significance in the milk industry worldwide, is characterized as a mammary gland inflammatory disorder which can be induced by physical or chemical agents, but majority of the causes are infectious and mainly due to bacteria that gain entry into the mammary gland (MG), multiply and develop toxins that are harmful to the mammary gland (Bonnetfont *et al.*, 2011; Schukken *et al.*, 2011; Jensen *et al.*, 2013). Mastitis includes in the most frequent and expensive diseases in the dairy enterprise, with losses linked to decreased milk production, rejected milk, early slaughter, veterinarian treatment, and operating costs (Thompson-Crispi *et al.*, 2014; Reshi *et al.*, 2015). The outbreak of a zoonotic disease is also likely, as is the emergence of microbial drug resistant bacteria (Bishop *et al.*, 2011). A huge array of pathogens induces mastitis and is epidemiologically grouped into contagious and environmental mastitis (Cervinkova *et al.*, 2013). Contagious pathogens are those for which the principle reservoir is the udders of the sick cows. They transmit from cow to another cow, mainly while milking, and with flare-ups of clinical episodes appear to develop in recurrent subclinical infections. *Staph. aureus*, *Strept. agalactiae*, *Corynebacterium bovis* as well as *Mycoplasma spp.* contain infectious pathogens. Conversely, environmental mastitis can be generally characterized as those intramammary infections caused by pathogens whose primary reservoir is the cow's living environment. Such environmental pathogens comprise of *E. coli*, *Strept. dysgalactiae* & *S. uberis*, *Klebsiella spp.*, and nearly all infections produced by them are of clinical and short-lived (Abebe *et al.*, 2016; Pal *et al.*, 2019). These mastitis-causing diverse pathogens induce different immune responses in the mammary gland, which is why, the host requires highly specific pathogen-dependent responses for protection (Thompson-Crispi *et al.*, 2014). We can also identify mastitis to be either clinical or subclinical (Wellnitz and Bruckmaier, 2012). Clinical mastitis may be specified by a sudden development, variation in the composition and presentation of milk, reduced production of milk, and the appearance of fundamental signs of inflammation in the contaminated mammary quarters. It is easily apparent and easy to identify. In comparison with that of sub-clinical manifestation, no visible symptoms are seen either on the udder or in milk, but the output of milk diminishes, and the somatic cells number increases. This is more frequent

and seriously affects the milking animals (Banger *et al.*, 2015; Tanamati *et al.*, 2019; Kirsanova *et al.*, 2020). For such reason as absence of any specific manifestation, the identification of sub-clinical form is a concern in milk animal management and in veterinary care (FAO, 2014).

Although several research has been done in dairy ruminants to understand the complex physiological and cellular activities that happen in the MG in reference to pathogens, the mechanisms of defence are still cryptic (Banos *et al.*, 2017). Globally, mastitis is accountable for a significant financial loss of USD 33 billion (Pal *et al.*, 2019). Notwithstanding strengthened management practices, the disease persists. Pal (2018) suggested that one of the biggest expenses to the dairy company globally was still acknowledged by the treatment and control of the disease mastitis.

### *Escherichia coli (E. coli) and Staphylococcus aureus (S. aureus)*

*E. coli* and *S. aureus* are the most predominant Gram-negative and Gram-positive bacterial pathogens that infect the mammary cells in dairy cattle respectively (Jensen *et al.*, 2013; Pal *et al.*, 2019).

*E. coli*, the cause of acute clinical mastitis, is amongst the most common environmental pathogens (Ju *et al.*, 2020). The *E. coli*-infected mastitic cows undergo serious systemic clinical symptoms; in the more severe cases, the disease can cause many deaths each year (Buitenhuis *et al.*, 2011; Lippolis *et al.*, 2018). This gram-negative bacterium constitutes the normal bovine intestinal flora and contaminates the environment through faeces, mostly bedding material. The cow is particularly vulnerable to coliform infections during the puerperal phase because the immune defence reaction is lower than normal at this time. Gram-negative bacteria produce endotoxin and the intramammary infection (IMI) normally results in extreme inflammatory reactions. Sometimes *E. coli* also can possibly cause subclinical mastitis, but these strains differ from those which cause clinical mastitis (Pal *et al.*, 2019). *E. coli* produces lipopolysaccharide (LPS) endotoxin, the most potent immunostimulant (Arenas, 2012; Xu *et al.*, 2019), and the key virulence factor eliciting clinical symptoms which is responsible for many of the inflammatory changes observed during coliform mastitis. The detection of LPS by Toll-like receptor 4

(TLR4) (Panigrahi *et al.*, 2014), mediated by a cluster of differentiation 14 (CD14), LPS Binding protein, and myeloid differentiation protein, is interconnected with the development of acute immune-response, enhanced SCC, activation of different leukocytes and immune related genes, release of cytokines (IL-6 and TNF- $\alpha$ ), chemokines (CXCL5, CXCL8, and RANTES) and leukocytic chemoattractant (IL-8) (Alnakip *et al.*, 2014; Thompson-Crispi *et al.*, 2014).

*S. aureus*, a gram-positive bacterium, goes on being the major cause of subclinical mainly but as well as, clinical mastitis worldwide (Murphy *et al.*, 2019; Pal *et al.*, 2019). The bacterium can quickly invade all types of cells in the MG and due to its intracellular localization in MG epithelial cells, it has a poor response to traditional antibiotic therapy. The intramammary infection induced with *S. aureus* progresses to chronic lifelong infection (Gilbert *et al.*, 2013). The infected cows act as a reservoir from which transmission to humans can occur both from physical contact such as while milking by hand method and consumption of unpasteurized milk. IMIs by *S. aureus* are therefore not only potentially harmful to the animal but also to humans. *S. aureus* isolated from intramammary infections produces many potential virulence factors including enterotoxins, hemolysin, hyaluronidase, and leukocidins (Pal *et al.*, 2019). Among the main immunostimulatory molecules, peptidoglycan (PGN) and lipoteichoic acid (LTA) have been proved to accelerate the development of inflammatory cytokines and chemokines by immune cells (Xu *et al.*, 2019), including monocytes and macrophages. In vitro trials have also shown that LTA itself can trigger the expression in mammary epithelial cells (MECs) of many cytokines, few of which are IL-1 $\beta$ , IL-8, IL-6 and TNF- $\alpha$  (Bougarn *et al.*, 2011). LTA has also been shown to greatly induce the release of CXCL1, CXCL2, CXCL3 and CXCL8 chemokines, which specifically target neutrophils (Kiku *et al.*, 2016) and it increases the expression of IL-17 driven immune response genes in MECs in vitro (Bougarn *et al.*, 2011). Toll-like receptor 2 (TLR2) plays a major part in the identification of LTA (Zhang *et al.*, 2016).

### Recognition of Invading Mastitis Causative Bacteria by Host Innate Immune System

Adequate immune functions are important for the

protection of the host against IMIs. MG immunity is the consequence of the integration between non-specific and specific protective factors, along with the anatomical characteristics of its gland and the defence factors of both cellular as well as humoral aspects (Schukken *et al.*, 2011). However, MG immune response differs through different lactation stages that occurs in dairy animals and is usually impaired via stress exposure and at the period of parturition and drying-off, thereby intensifying susceptibility to mastitis (Moosavi *et al.*, 2014; Wu *et al.*, 2020).

Innate and adaptive immunity are two essential components of MG host defence to defend against infections. The adaptive immune system (AIS) responds more competently to previously exposed threats yet responding to new threats is sluggish. However, the innate immune response is the very first line of defence towards the bacteria after violating the physical boundary of the teat canal and prior to when the AIS comes into action (Sordillo, 2018). This mechanism is implicated in a variety cascades of intracellular signal transduction that induce acute upregulation of many other innate immune elements, namely different leukocytes, molecules of adhesion and cytokinesis (Prince *et al.*, 2011; Wellnitz *et al.*, 2011).

The activation of an efficient and timely innate immune response (IIR) largely relies on recognising the infectious agent. MG IIR is prompted as pattern recognition receptors (PRRs) bind to pathogen-associated molecular patterns (PAMPs) considered bacterial motifs (Wellnitz and Bruckmaier, 2012). These motifs may be revealed during a microorganism's multiplication or degradation. A signalling transduction cascade is triggered by activation of these PRRs which contributes a vital part in controlling multiple signals and coordinating the expression of effector response genes, like cytokines, thus further stimulating local and systemic immune responses (Schukken *et al.*, 2011; Stevens *et al.*, 2011). Increases in PRR expression in the epithelia and tissues of affected bovine MGs and TLRs are the well-versed studied bovine PRRs and identify a large spectrum of PAMPs (Alnakip *et al.*, 2014).

As pathogens enter the udder lumen via the teat channel, both immune and non-immune cells sense them, and the production of chemoattractant follows. Consequently, neutrophils migrate from the bloodstream to the area of

infection. These cells possibly can generate phagocytes and exercise bactericidal activity by production of potent oxidative products (Prince *et al.*, 2011). This huge recruitment of udder neutrophils brings about a drastic rise in the somatic cells count in the milk (SCC). Consequently, SCC were been employed widely as simple to use method for recognising mastitis and distinguishing among chronically infected and healthy or non-infected animals (Rainard *et al.*, 2018).

There is ample evidence that the reaction of the host to IMI is subject to genetic control, but the underlying genetics for the resistance remains largely unclear. New progress in high throughput technology (RNA-Seq), which now facilitates the analysis of several thousands of infection-associated genes expression, has offered considerable insight into the host response to pathogens. Gene expression profiles for challenged mammary tissue (Kosciuczuk *et al.*, 2017), milk cells (Wickramasinghe *et al.*, 2012) and peripheral blood leukocytes (Wang *et al.*, 2020) were studied using high throughput techniques such as RNA-Seq.

### **RNA Sequencing (RNA-Seq)- A Significant Method to Analyse Mastitis Associated Gene Expression**

RNA molecules are highly vital components of all living cells. The primary aim of RNA investigation is to comprehend the identity and quantification of each RNA molecule in each cell under a particular condition. Most of what we know about RNA comes from biochemical examination of which a few numbers of specific molecules are studied. High-throughput approaches have also emerged which enable large-scale interrogation of RNA sequences (Hrdlickova *et al.*, 2016).

Early gene expression analysis methods such as the expressed sequence tag (EST) technique described during 1990s analysis showed both the sequence and quantification of corresponding RNAs through partially sequencing complementary DNA (cDNA) clones. As in early 1990s, EST data played a crucial role. The high cost of the sequencing process, however, narrowed down the use of such expression processing. The Serial Analysis of Gene Expression (SAGE) technique was then introduced by significantly reducing the high cost of expression analysis per gene. However, with the advent of DNA microarray technology in the mid to later 1990s,

replaced EST and SAGE techniques for gene expression research, primarily due to their much greater affordability for large-scale studies (Farkas *et al.*, 2015; Hrdlickova *et al.*, 2016). On the contrary, microarrays allow only the relative quantitation of transcripts and often depend on a properly annotated genome. It can only analyse transcripts of previously known, with no capability of recognizing alternative splice sites or new exons. Subsequently, the Next Generation Sequencing (NGS) or so-called deep sequencing *i.e.* RNA-Seq, a paradigm shift in bio-medicine due to its ability to produce an incredible amount of data in a relatively short period has since then rapidly transformed RNA research. RNA-Seq provides low background signal, much more quantifiable and not limited to genomic sequences as contrast to DNA microarray-based methods (Nookaew *et al.*, 2012; Adiconis *et al.*, 2013; Van Dijk *et al.*, 2014; Li *et al.*, 2014; Han *et al.*, 2015).

RNA-Seq has emerged as a widely used and important approach for characterizing transcripts, analysing gene expression and detecting RNA biogenesis and metabolism, offering powerful tools to uncover molecular pathways in development, differentiation and disease (Costa-Silva *et al.*, 2017). RNA-Seq helps us to examine and discover transcriptome, the complete cellular content of RNAs, namely mRNA, rRNA and tRNA are few examples. Understanding the transcriptome is fundamental if we are to link information about our genome to the functional protein expression. RNA-Seq can show us which genes are switched on in a cell, their level of expression and when they are activated or shut (Ozsolak and Milos, 2011). Initially, the technique converts the RNA population to cDNA fragments (a cDNA library). This enables the RNA to be integrated into the NGS procedure. Then NGS analyses the library, generating short sequences that match to either one or both ends of the fragment. Subsequently, reads can be matched with a reference genome and assembled to generate an RNA sequence map that covers the transcriptome (Zhao *et al.*, 2015), and the levels of expression for each gene are calculated.

Many tools and pipelines for the research of differential gene expression were been built from these sequenced data with the popularisation of RNA-Seq technology. Numerous studies have also been conducted lately on the transcriptome analysis of RNA-Seq-based mastitis in cattle (Chen *et al.*, 2016; Fang *et al.*, 2017; Lai *et al.*, 2020; Wang *et al.*, 2020). To state the obvious, RNA-Seq

is excellently known as the superior microarray alternative and is possibly to persist as the favoured available option setting the foundations for modern genetic investigations.

Some studies related to mammary transcriptome profiling performed through RNA-Seq are explained briefly in table 1.

Mastitis is a result of the consequence of several cellular and soluble factors that operate together to remove invading microorganisms. Therefore, it may be necessary to integrate more biological information related to the studied complex traits to correlate the flow of biological information underlying this trait, which will supplement us find the appropriate genomic characteristics that

**Table 1:** Few research conducted to provide a transcriptome profile of the bovine mammary gland via RNA-Seq analysis

References	Works done so far
Wickramasinghe <i>et al.</i> 2012	Characterize the detailed bovine milk transcriptome in Holstein cows and demonstrated the capacity of milk somatic cells to respond to various molecular functions according to the animal's biological needs. As during transition lactation, a number of 16,892 genes were expressed, 19,094 genes during peak lactation, whereas, during the late lactation about 18,070 genes were revealed.
Chen <i>et al.</i> 2016	Demonstrated that mammary stromal fibroblasts derived from mastitic cows show inflammation-specific changes compared to those without mastitis from dairy cows and reflect their capacity for inflammation. And significantly enhanced levels of IL6 and TNF- $\alpha$ protein were detected by inflammation-associated fibroblasts (INFs) compared to normal fibroblasts. A total of 727 differentially expressed genes were discovered, 372 and 355 of which were up and down regulated in INFs, respectively.
Fang <i>et al.</i> 2017	The research linked genome-wide association analysis (GWAS) to IMI-relevant RNA-Seq data and provided a framework for the integration of multiple data layers to give a clear perspective about the underlying genetic architecture of complex features, such as mastitis resistance and production of milk in dairy cattle. And genomic evidence has been given that the genes in the mammary gland which reacted to IMI were more associated with mastitis prevention than with the generation of milk.
Luoreng <i>et al.</i> 2018	RNA-Seq was employed to screen the miRNA gene expression profile post-infection with <i>E. coli</i> at different point of times. A total of two hundred miRNAs articulated differentially were found at different times. The study is suggestive that these RNAs may regulate the mastitis development in dairy cows via various signal transduction pathways.
Xu <i>et al.</i> 2019	Investigated variations in the global transcriptional response in regard with bovine mammary epithelial cells (BMECs) to LPS and LTA. In the LPS-treated group, they found 100 differentially expressed genes (DEGs) with 95 up-regulated and 5 down-regulated genes, while in the LTA-treated group, 24 DEGs have 12 up-regulated and 12 down-regulated genes in contrast to control.
Han <i>et al.</i> 2020	Transcriptome sequencing was conducted in healthy Chinese Holstein mammary glands to investigate the differentially expressed genes associated with inflammation and immunity. A number of 446 DEGs were found in Chinese Holstein's late-lactation mammary tissue (high SCC period) and during peak lactation (lower SCC period). Functional review showed 50 immunity and inflammation related DEGs, such as TLR2, IL-1, TNF- $\alpha$ , CXCR1, CXCR2, CXCL8, SLC11A1 and TNFAIP3, TRAF6, RIP1.
Lai <i>et al.</i> 2020	Discovered that in modified and regular milk samples, 2-downregulated and 23-upregulated genes exhibit differential patterns of expression. 3 additional miRNAs has been found to be significantly associated with bovine mastitis.
Wang <i>et al.</i> 2020	They conducted a study with the major goal for discovering candidate transcriptional blood-based biomarkers for <i>S. aureus</i> -induced bovine mastitis and offers new depth analysis of disease resistance genetics. 5 genes (EPOR, IFNL3, CCL26, IL9 and IL26) were shown to be enriched with cytokine-cytokine receptor pathway linked with immune response in 4 biological pathways, which may serve as molecular markers in breeding programmes.

are highly enriched for causal variants (Fang *et al.*, 2017). High throughput RNA-Seq has become one such important instrument for resolving complex biological issues by simultaneously evaluating thousands of genes expression in the tissue. Attribution of gene expression patterns would provide insight into their encoded proteins' biological roles and interacting proteins networks that influence cellular responses to extracellular stimuli.

The assessment of these studies would certainly require a lot of effort to make highly concrete conclusions about the functional utility of this knowledge to develop the standard approach of treatment or control of the animal disease. Moreover, cattle diseases like mastitis have a large effect on the immune reaction expression and other genes.

### Prospects

The diversity in the prevalence and abundance of mastitis-causing organisms and the variation in host responses make mastitis a complex disease that continues to be a burden on the dairy sector. Generally, a disease management can be approached either of the two ways, by therapeutic approach that incorporates the high cost of veterinary diagnosis and services, antibiotic treatments, use of vaccine, etc., and the other way is via genetic approach of breeding for mastitis resistance. The earlier approach has instead contributed to the proliferation of numerous drug resistant strains of causative species (Chandrasekaran *et al.*, 2014; Algamal *et al.*, 2020) and failure of the vaccine to mount immunity against enormous mastitis-causing etiological agents (Mata, 2013), whereas the later approach was viewed to be an economic and prophylactic solution comparatively (Karthikeyan *et al.*, 2016).

The initial step for such a strategy would be to correctly classify DEGs in recognising phenotypic variation under complex mastitis-related conditions. The discovery of the DEGs among the susceptible and resistant individuals offers key information for identifying candidates or marker for the underlying genetic basis of mastitis susceptibility or resistance, this lays the foundation for further modern genetic and mechanistic studies or approaches including genomic selection, marker-assisted selection or genome-wide association studies (Bonnefont *et al.*, 2011; Thompson-Crispi *et al.*, 2014; Fang *et al.*, 2017; Yan *et al.*, 2020). To cite an example, Usman *et al.* (2017) also conducted a study suggesting that cytokines: IL-17F and

IL-17A could be effective mastitis resistance candidate genes, and in both milk and dual-purpose animals, large SNPs may deemed as useful genetic markers to manage mastitis. Likewise, the peripheral blood leukocyte (PBL) obtained from *S. aureus* positive mother daughter pairs and *S. aureus* control mother daughter pairs were subjected to transcriptomic evaluation and characterized using RNA-Seq by Wang *et al.* (2020). As follows, differential gene expression was assessed in a consecutive generation of dairy cattle: revealing contrasting DEGs. The study suggested that the genes outlined in the analysis may qualify as biomarkers of mastitis expression and may also include variations in the sequence which could be used for genetic enhancement of dairy cattle in mastitis resistance.

MG immune response is complicated as mastitis is subjected to a wide variety of infectious agents with various pathogenic mechanisms requiring complex pathogen-linked immune response. And the prevalence in dairy cattle is lesser with improved and balanced immune response and can be identified using high immune response (HIR) technology. Increased immune response is often characterised by significantly higher immunisation response, higher production of milk and colostrum, strengthened animal welfare, and higher quality of food to sustain the increasing population. As immunity is a major fitness trait, beneficial correlations with survival and reproduction are also widely noted (Thompson-Crispi *et al.*, 2014). Strengthened immune response can be achieved by selecting disease resistance in livestock, which is taken into consideration to be the most relevant prophylactic strategy for improving animal welfare.

Therefore, in the near term, the task is to get a full discovery and detailed study of a significant systemic response in the mammary gland that affect the interaction among the host and various etiological agents. As it is expected that the understanding of RNA sequencing data and the position of functional SNPs or biomarkers in the host and the pathogen would connect the dots among the immune function and the relevant genes with potential to enhance resistance to pathogens specifically. The expertise of host-pathogen relationship in mastitis will probably be essential for the formation of new therapeutics and for performing genetic selection approach for mastitis resistance. The inclusion of mastitis resistance during the selection programs in Scandinavian countries has resulted in a greater economic response to selection (Sulabh, 2016). Such strategy can

result in animal herd developing with increased resistance or tolerance to mastitis.

## CONCLUSION

Mammary gland inflammation (mastitis) is one of dairy cattle's most common disease with an alarming economic loss in dairy industry worldwide. Genetic control of selection of immune response and disease tolerance is considered as one of the most economic and prophylactic measure to manage the disease. Mastitis tolerance, however, results from fine coordination of immune as well as inflammatory responses within a complex network of cell-gene interactions. Therefore, it is important to provide the scientific basis for generating immune intervention tools that improve the outcome of infections and defend against damages in bovine host mammary gland and the image of a dairy industry. With the rapid technological advances, given opportunity to study in more depth the expression of genes than ever, namely RNA-Seq have contributed to our understanding of the transcriptional characteristics and profile providing a basis for extending research scope to develop new targets for handling mastitis.

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