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Trends in Diagnosis and Control of Bovine Mastitis: A Review

[1]Rajib Deb, [2]Amit Kumar, [4]Sandip Chakraborty, [3]Amit Kumar Verma, [2]Ruchi Tiwari, [5]Kuldeep Dhama, [1]Umesh Singh and [1]Sushil Kumar
[1]Animal Genetics and Breeding, Project Directorate on Cattle, Indian Council of Agricultural Research, Grass Farm Road, Meerut, 250001, Uttar Pradesh, India
[2]Department of Microbiology and Immunology,
[3]Department of Veterinary Epidemiology and Preventive Medicine, Uttar Pradesh Pandit Deen Dayal Upadhayay Pashu Chikitsa Vigyan Vishwa Vidyalaya Evum Go-Anusandhan Sansthan (DUVASU), Mathura, 281001, Uttar Pradesh, India
[4]Veterinary Officer, Animal Resource Development,
Department of Pt. Nehru Complex, Agartala, 799006, Tripura, India
[5]Division of Pathology, Indian Veterinary Research Institute, Izatnagar,
Bareilly Uttar Pradesh-243122, India

Abstract: Mastitis (inflammation of mammary gland) is a most devastating disease condition in terms of economic losses occurring throughout the world. The etiological agents may vary from place to place depending on climate; animal species and animal husbandry and include wide variety of gram positive and gram negative bacteria; and fungi. They may be either contagious viz. *Staphylococcus aureus*; *Streptococcus agalactiae* or environmental viz. *S. dysgalactiae*, *S. uberis*, *Corynebacterium bovis* and Coagulase negative *Staphylococcus*. Conventional diagnostic tests viz. California Mastitis Test (CMT); R-mastitest and Mast-O-test methods are applied under field conditions; whereas somatic cell count and Bulk Tank Somatic Cell Count (BTSCC) are useful for early mastitis detection and detection of sub clinical or chronic mastitis respectively. *In vitro* culture based diagnosis require further study as they can detect only viable cells. The advent of Polymerase Chain Reaction (PCR) technology along with its various versions like multiplex and real time PCR has improved the rapidity and sensitivity of diagnosis. Circulating micro RNA (miRNA) based diagnosis; immune assay and proteomics based detection along with biochips and biosensors prove to be asset to diagnosticians for advanced diagnosis of this economically important condition. Improvement of milking hygiene; implementation of post-milking teat disinfection; regular control of the milking equipments; implementation of milking order; Improvement of bedding material are the general measures to prevent new cases of mastitis. The use of antibiotics (intramammary infusions; bacteriocins) and herbs (*Terminalia* spp.) are important for prophylaxis and therapeutics. Vaccines viz. cell based; Recombinant (staphylococcal enterotoxin type C mutant) or chimeric (pauA); live (*S. uberis* 0140J stain based) and bacterial surface extract based; DNA-based and DNA-protein based have greatly aided in management of bovine mastitis. Quorum sensing and disease resistant breeding using novel biomarkers viz. toll like receptors (TLR) 2 and 4, interleukin (IL) 8; breast cancer type 1 susceptibility protein (BRCA1) and calcium channel voltage-dependent alpha 2/delta sub unit 1 (CACNA2D1) are also indispensable. This mini review gives an overview of all these different aspects that act as trend setters as far as the diagnosis and control of bovine mastitis is concerned to help the diagnosticians; epidemiologists and researchers not to remain ignorant about this grave condition.

Key words: Biochips, biosensors, CAMP, GapC, lacticin, mastitis, proteomics, SCC, miRNA, multiplex PCR, quorum sensing

INTRODUCTION

Mastitis (inflammation of mammary gland) is a most devastating disease condition in terms of economic losses occurring throughout the world (Kumar *et al*., 2010). Due to the involvement of multiple etiological agents it always remained a challenge to veterinarian all over the globe (Vashney *et al*., 2012). Depending upon the climatic condition, animal species and animal husbandry practices etiological agents may vary place to place and case to

Corresponding Author: Rajib Deb, Scientist, Animal Genetics and Breeding, Project Directorate on Cattle, Indian Council of Agricultural Research, Grass farm Road, Meerut, Uttar Pradesh, 250001, India

case. That is the region that behind the isolation of largest number of pathogens in a single disease i.e. more than 135 is from the cases of mastitis (Awandkar *et al*., 2009; Kumar *et al*., 2010). Thus the control and prevention of mastitis is a challenge and despite of the continuous efforts it is a cause behind the severe economic losses to dairy industry (FAO, 2005). Rendering the animal production less and diminution of milk quality and quantity can be the greatest hurdle in true sense for the dairy and livestock owners along with dairy industry. Moreover, different forms of mastitis viz., subclinical, clinical, acute make it a Pandora box for microbiologists (De Vliegher *et al*., 2012). The involvement of bacteria, virus, fungi and protozoa make mastitis an omnibus of most pathogens. In spite of advancement in scientific skill and management (Amin *et al*., 2011), milk remains an excellent growth medium for the growth of almost all kind of microbes (Kumar *et al*., 2010). The body temperature of the animal also supports the growth of microbes and that is the reason due to which almost all the known pathogens have been isolated or reported from the cases of mastitis. The presence of all these always have alteration in physical, physiological conditions of animals and also change the color, texture and composition of milk (Sharma *et al*., 2007) which can be used as markers for the disease diagnosis (Jiusheng *et al*., 2008; Syring *et al*., 2012) and can assist in the prevention or timely control of pathogens, avoiding the severe damage to udder and losses in the form of productivity and ultimately economically (Radostits, 2007). This review imparts some light on such aspects which can help in better understanding the mastitis with possible path for the diagnosis along with prevention and control of disease.

Microbiology of bovine mastitis: The etiology of bovine mastitis can be classified into contagious pathogens such as *Staphylococcus aureus*, *Streptococcus agalactiae* and environmental pathogens viz. *S. dysgalactiae*, *S. uberis*, *Corynebacterium bovis* and Coagulase negative *Staphylococcus* (Reyher *et al*., 2012). Counting of somatic cell rises>200,000 cells mL^{-1} have been seen in cow milk during bacterial infection and the counting parameters vary between kind of bacterial infection (Dohoo and Meek, 1982). Schepers *et al*. (1997) studied that, somatic cell count of *S. aureus* is greater than *Corynebacterium bovis* infection. Malinowski *et al*. (2006) reported that, somatic cell count of coagulase-negative staphylococci (CNS), *Staph. aureus* and *Streptococcus* sp. were 200,000 to 2,000,000 of SCC mL^{-1} (59.6%), = 10 million mL^{-1} in intramammary infections by *Arcanobacterium pyogenes* (95.5%), = 5 million mL^{-1} was connected with infections caused by *Prototheca* sp. (64.5%), yeast-like fungi (60.2%) and *Streptococcus* sp. (55.1%). *S. aureus* (76.2%), CNS (84.2%), Gram-positive bacilli (72.4%) and *Corynebacterium* sp. (83.2%). Among the isolation of bacterial pathogens majority of cases are reported due to *Staphylococcus* sp., *E. coli*; *Streptococcus* sp. and *Bacillus* sp.,; *Kliebsiella* sp., *Proteus* sp., *Pseudomonas* sp., *Micrococcus* sp. and *Salmonella* sp. (Atyabi *et al*., 2006; Sahay *et al*., 2006; Hawari and Al-Dabbas, 2008; Kumar *et al*., 2010). The presence of bacterial pathogens either in monoculture or with mixed etiology vary from case to case and stage of disease condition. In most of the acute cases monoculture is recovered (Kumar *et al*., 2010).

DIAGNOSTIC OVERVIEW OF BOVINE MASTITIS

Conventional field tests: California Mastitis Test (CMT) is a simple cow-side indicator test for subclinical mastitis by somatic cell count estimation of milk which allows the DNA in those cells to react with the test reagent, forming a gel (Middleton *et al*., 2004; Whyte *et al*., 2005). The reaction is scored on a scale of 0 (where mixture remains unchanged) to 3 (solid gel forms) with a score of 2 or 3 being considered a positive result (Anonymous, 2008). R-mastitest is used as a indirect test for cow's mastitis diagnosis and is based on the principle of CMT (Sargeant *et al*., 2001). To detect the relationship between milk electrical conductivity and its salt and lactose concentration Mast-O-Test method (a specialized one) is used (Musser *et al*., 1998). Other methods described are Portacheck (esterase-catalysed enzymatic reaction), Fossomatic SCC method, electrical conductivity and pH tests etc. (Viguier *et al*., 2009). It is a test which is not very costly and nontechnical persons or laymen can use it as routine test for the dairy animals. The implication of test will surely improve management and prevent the chances of mastitis (Pitkala *et al*., 2005).

Somatic cell counting: Somatic cells are the epithelial (25%) and leukocytes (75%) cells secreting through milk. If inflammation i.e., mastitis occur, somatic cells number also become higher and it is due to migration of more neutrophils in the milk which is around 90% (Harmon, 1994). Measurement of somatic cell in the milk samples are referred as Somatic Cell Count (SCC). There various factors related to occurrence of bovine mastitis like level of infection (Sharma, 2003); stage of lactation (Dohoo and Meek, 1982); Age and breed (Beckley and Johnson, 1966; Singh, 2002); parity as well as season and stress (Skrzypek *et al*., 2004; Khate and Yadav, 2010; Smith *et al*., 1985); Diurnal variation (White and Rattray,

1965) and milk transport management (Gonzalo *et al*., 2003). SCC if is lower than 1×10^5 cells mL^{-1}, indicate normal milk and while during infection it can rise to above 1×10^6 cells mL^{-1} (Bytyqi *et al*., 2010). Thus somatic cell count can be referred as an indicative test for early diagnosis of mastitis and any alteration in cell count can be correlated with the presence of potent pathogen. Bulk Tank Somatic Cell Count (BTSCC) has to be reported on bimonthly basis as a measure of milk quality. BTSCC less than 2×10^5 cells mL^{-1} indicate a minimum level of infection. A series of BTSCC's over 5×10^5 cells mL^{-1} indicate a problem with sub clinical or chronic infection (Schepers *et al*., 1997).

***In vitro* culture based diagnosis:** *In vitro* culture regarded as gold standard test for mastitis (Pyorala, 2003).Swab of Milk samples can be taken for bacterial, viral and fungal culture in a specific media and further microbiological/biochemical test applied for specific detection of bacteria viz. coagulase tests for *Staphylococcus* (Rajeev *et al*., 2009). The main draw back with bacterial culturing is that, they need specific medium and time consuming. Moreover, the isolation and identification of viral etiological agent is cumbersome and facilities are also a limitation. Virus isolation is very tedious from the cases of chronic infections or the cases with secondary invasions. In contrast fungal pathogens can be isolated easily in routine microbial media but it is time consuming as fungi can take weeks together to grow in laboratory media. It is important to note that culture is capable of detecting only viable cells and thus the clinical relevance of culture negative results requires further study (Koskinen *et al*., 2010).

PCR based diagnosis: Compared with bacterial culture methods, PCR based detection from directly mastitis milk samples are less time consuming (Amin *et al*., 2011). Another main advantage of PCR based assay is based on DNA and thus no matter of live or dead organisms which is crucial point for culture based detection but one disadvantages is that PCR detect lower number of organisms compare to culture methods (Yamagishi *et al*., 2007; Madico *et al*., 2000; Riffon *et al*., 2001). Various PCR based tools has been demonstrated for detecting microbes in mastitis milk samples viz .165 and 165-235 spacer rRNA genes based touchdown enzyme time-release-PCR for detection and identification of *Chlamydia trachomatis*, *C. pneumoniae* and *C. psittaci* (Madico *et al*., 2000); detection of *Staphylococcus* spp., *Escherichia coli* and *Streptococcus* spp., (Amin *et al*., 2011); a rapid PCR test for identification of *Streptococcus agalactiae* by 16S-23S rRNA intergenic spacer region (ISR) amplification (Jiusheng *et al*., 2008); *Staphylococcus aureus* genotype B (GTB) detection (Syring *et al*., 2012).

Multiplex PCR based diagnosis: Mastitis is known for the involvement of multiple etiological agents and many times failure of the treatment is due to failure of real damage causing orgamism. Mostly fastidious etiological agents remain untraced and for all such conditions multiplex PCR can be a boon for the veterinarian as multiplex PCR can identify multiple pathogens in a single reaction and at a same time (Phuektes *et al*., 2001).The main drawback with multiplex PCR is that there is competition between different sets of primers for PCR substances like dNTPs and *Taq* polymerase which reduces the sensitivity (Amin *et al*., 2011).Multiplex PCR also used for diagnosis of multiple pathogens in bovine mastitis milk samples (Phuektes *et al*., 2001, Amin *et al*., 2011).

Real time PCR based detection: Real-time PCR based assay is an alternate to in vitro culture for detecting bacterial pathogens in milk samples. Taponen *et al*. (2009) detected 11 *Staphylococcus* spp. (Staphylococci other than *Staphylococcus aureus*); 10 *Streptococcus uberis*; 2 *Streptococcus dysgalactiae*; 6 *Corynebacterium bovis*; 3 *Staph. aureus*; 1 *Escherichia coli*; 1 *Enterococcus* and 1 *Arcanobacterium pyogenes* from mastitis milk samples using real time PCR based detection. However, the presence of costly instruments and consumables make it difficult to afford particularly in developing countries (Koskinen *et al*., 2009; Rajeev *et al*., 2009).

Circulating miRNA as novel diagnostic tools: MicroRNA/miRNA are 22 nucleotide long non coding RNA act as transcriptional and post-transcriptional regulation during expression of genes (Chen and Rajewsky, 2007). They are complimentary to the 3'UTR region of mRNA and thus participate in gene regulation (Wang *et al*., 2004). Bioinformatics analysis revealed that 89 putative miRNA target sites present in 18 mastitis candidate genes. It was also experimentally proved already that Bta-mir-142* have target site on SAA3 mastitis candidate gene expressed in bovine mammary tissues (Gu *et al*., 2007).

Immune assay: ELISA based diagnostic already developed for *S. aureus* (Fox and Adams, 2000), *Listeria monocytogenes* (Kalorey *et al*., 2007), magnetic-bead-based ELISA for detecting Staphylococci using beads coated with an anti- *S. aureus* monoclonal antibody (Yazdankhah *et al*., 1998). Immunoassays also used for detecting inflammation-related biomarkers present in the

milk at different stages of sub-clinical mastitis viz. immune assay based detection of various biomarkers like heptoglobulin (Hiss *et al*., 2004), acute phase protein (Eckersall, 2007) etc.

Proteomics based detection: Advancement in proteomics tools for examples two-dimensional gel electrophoresis (2D-GE) and mass spectroscopy (MS) (Lippolis and Reinhardt, 2005; Smolenski *et al*., 2007) helped to identify various protein expressed during mastitis. These methods can be applied to detect the marker proteins from the cases of mastitis particularly from the acute, subacute and chronic mastitis. It stems largely from the need of to better characterize the mechanism of the disease and as measure of early detection and drug efficacy (Boehmer, 2011).

Biochips for detecting mastitis: "Biochips" are so called as 'laboratory-on-a-chip' or microfludics having the capacity to use as a diagnostics (Garcia-Cordero and Ricco, 2008) are already used for the detection of bovine mastitis viz. Lee *et al*. (2008) developed a biochip that integrated DNA amplification of genes that are specific for seven known mastitis-causing pathogens; a microfluidic device that incorporated solid-phase extraction and NASBA has been reported for the identification of low numbers of *E. coli* (Dimov *et al*., 2008) etc.

Biosensors for detecting bovine mastitis: Biosensors are biological sensors which use bio-receptors like-antibody, nucleic acid, enzymes etc. and produce a signal after combination with transducers. Nowadays some of the biosensors already developed for detecting bovine mastitis for examples For example, an electrobiochemical sensor developed by Pemberton *et al*. (2001) and a competitive biosensor assay using surface plasmon resonance developed to discriminate between sub-clinical mastitic and non-mastitic milk (Akerstedt *et al*., 2006).

PREVENTION AND CONTROL

Prevention is better than cure is the phrase that perfectly defines the condition mastitis, as there is much alterations viz. damage to udder alveoli, mesenchyma, teat canal, teat itself which cannot be cure flawlessly. These are the damages occurring as squeal to mastitis. Good hygienic practices and better animal husbandry way of animal handling can reduce the chances of mastitis (Kumar *et al*., 2010). Most of the cases of mastitis are due to the injury of the udder followed by microbial infection and these can be avoided and even if these occur accidentally, treatment should be rapid and regular. The portal of entry of pathogens is mainly through open teat canals and high yielding animals with soft opening and delayed closing of teat canal during the milking, or the delayed milking leading to dripping of milk from teats might the route of infection from soil, contaminated water or litter. These all can be handled by good management and cleanliness in sheds. Use of antiseptics post milking and at the opening of teat also reduces the chances of microbial entry, thus considered as an effective management practice for prevention of contagious mastitis (Olde Riekerink *et al*., 2012). Timely and routinely practices of disinfectants in the shed and paddocks always reduce the incidence of mastitis. Regular screening of milk and milk samples always reduce the number of infected animals. Commonly used practices to strain milk, observation of color and consistency improves the chances of early diagnosis of mastitis. As such no effective vaccine is available against all possible pathogens due to multiple etiology, however various vaccines have been attempted against bacterial pathogens with mixed success. Measures aiming at preventing new cases of mastitis include breeding; fly control; optimal nutrition; improvement of milking hygiene; avoidance of inter-sucking among young ones; implementation of post-milking teat disinfection; regular control of the milking equipments; implementation of milking order; improvement of bedding material (Shkreta *et al*., 2004; Calzolari *et al*., 1997; Fontaine *et al*., 2002; Chang *et al*., 2008; Nielsen, 2009; Yin *et al*., 2009; De Vliegher *et al*., 2012).

Antibiotic and herbal treatments: During the clinical mastitis cases the cow is first milked out and then introduced with intramammary infusion of antibiotics. To control *Streptoccocus agalactiae*, all four quarters of positive cows need to be treated with an appropriate commercially marketed intramammary antibiotic (Erskine, 2001; Sharma *et al*., 2007). Again, intramammary treatment with a commercially available penicillin and novobiocin products give better results against *Staphyloccocus aureus* (Owens *et al*., 1997). Oxytocin treatment and frequent milking is recommended as an accessory therapy for subacute and acute coliform mastitis (Leininger *et al*., 2003). The treatment of mastitis is mostly based on hit and trial, it makes condition beyond repairable. Involvement of multiple etiological agents makes it necessary to perform antibiotic drug sensitivity prior to select the final line of treatment (Kumar *et al*., 2010). There are variation of reports of drug sensitivity patterns of bacterial pathogens from different geographical region and animal species. Use of two nisins viz. Ambicin (nisin A) with germicidal activities against *Staph. aureus*; *S. agalactiae*; *S. uberis*; *Klebsiella pneumoniae* and *E. coli* and nisin Z are widely accepted. A new bacteriocin produced by *Lactococcus lactis* spp. *lactis* DPC3147 is also found to be effective against a wide range of gram positive

bacteria. Additionally lactin NK34 (partially purified from *lacticin* NK34) has *in vivo* preventive and therapeutic effects on mouse infection model using mastitis pathogens (Espeche *et al*., 2009; Bogni *et al*., 2011). Treating sub clinical mastitis with antimicrobials during lactation is not practiced because of high cost of treatment and poor efficacy. Herbal therapy is gaining much attention nowadays due to increased drug resistance and in this regard uses of *Terminalia chebula* and *Terminalia belerica* are quiet significant (Sahay *et al*., 2006; Hawari and Al-Dabbas, 2008; Pyorala, 2009; Awandkar *et al*., 2009; Kumar *et al*., 2010; Vashney *et al*., 2012).

Vaccines and vaccination strategy: Some of the vaccine already in field trial against control of bovine mastitis include: inactivated, highly encapsulated *Staphylococcus aureus* cells; a crude extract of *S. aureus* exo polysaccharides; Staphylococcus aureus CP5 whole cell vaccine; inactivated and unencapsulated *Staph. aureus* as well as *Streptococcus* spp. Cells based vaccine developed for contolling bovine mastitis (Calzolari *et al*., 1997; Camussone *et al*., 2013); recombinant staphylococcal enterotoxin type C mutant vaccine (Chang *et al*., 2008); recombinant *Streptococcus uberis* GapC or a chimeric Christie Atkins Munch-Petersen (CAMP) antigen; pauA; live *Strep. uberis* 0140J stain and bacterial surface extract (Finch *et al*., 1997; Fontaine *et al*., 2002); DNA vaccine containing clumping factor A of *Staphylococcus aureus* and bovine IL18 (Yin *et al*., 2009); DNA-Protein vaccine against *Staphylococcus aureus* (Shkreta *et al*., 2004) and so on.

Quorum sensing: It is the regulation of gene expression in response to fluctuations in population density of cells based on the principle of release of auto inducers by bacteria that increase in concentration as a function of cell density. Several of them have been intensively studied in *Staph. aureus* which when interact with specific receptors activate the transcriptional control system consisting of the genetic elements *agr* and *sar*. Similar studies on *Strep. uberis* has shown the presence of QS genes such as *luxS* and *comEA* responsible for group behaviour. As these two bacteria in particular have the ability to grow in infected tissues on biofilms they develop an innate resistance to almost all therapeutic agents. The difficulties in treating recurrent infections may thus be related to this pathogen ability and will ultimately allow better use of quorum sensing to find effective and appropriate treatment protocols (Kerr and Wellnitz, 2003; Waters and Bassler, 2005; Novick and Geisinger, 2008; Moore, 2011).

Disease resistant breeding: With the advancement of molecular/ quantitative genetics, marker-assisted selection (MAS) are one of the novel ways for selecting disease resistant breeds for mastitis. Candidate genes can be chosen on the basis of known relationship between physiological /biochemical processes as well as production traits so called Quantitative Trait Loci (QTL) (Deb *et al*., 2012). Various novel genes has been identified and their association analysis with SCC revealed mastitis resistant biomarkers viz. TLR2 (Zhang *et al*., 2009), TLR4 (Deb *et al*., 2013), IL8 (Chen *et al*., 2011), BRCA1, (Xu *et al*., 2011), CACNA2D1 (Yuan *et al*., 2011) and so on.

CONCLUSION AND FUTURE PERSPECTIVES

In general mastitis is a condition which is at present among the most severe damage causing conditions to dairy industry. The economic losses due the conditions are beyond repair and this is due to late and improper diagnosis of main etiological agent. Complicity of disease is primarily the reason behind the failure of diagnosis. Although there are many advanced tests available for the diagnosis but the core issue is early and effective diagnosis as the losses occurs so quickly that the delay of few hours can be the loss of completer teat or udder. In such conditions mastitis can be handled best by two means, first one through continuous monitoring with routine examination of physical condition of udder, milk and examining the quality of milk. Secondly use of disinfectants and available vaccines in endemic areas on regular basis. Establishment of protein expression profile for early disease diagnosis; use of bacteriocins for treatment and disease resistance breeding aiding in mastitis management are quiet noteworthy. The absence of single shot vaccination and treatment therapy however still possess the challenge to veterinarians and research community. Lot of training and awareness is required in particular to under developed and developing country animal owners to make them aware of good hygienic practices and routine or regular monitoring of disease. All control tools must be developed with the ultimate aim to manipulate the immune status of the animal and to reduce the carrier state. Moreover, establishment of testing laboratories and skilled and trained laboratory personnels for testing is another part of the picture.

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