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Technological Advancement in Diagnosis of Bovine Mastitis: An Overview

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ABSTRACT

Mastitis caused by infectious pathogens is still considered a devastating condition of dairy animals affecting animal welfare as well as economically incurring huge losses to the dairy industry by means of decreased production performance and increased culling rates. The inflammation of a cow's udder and mammary glands, known as bovine mastitis, is typically brought on by bacterial infections. The clinical and subclinical signs and symptoms of the disease serve as the foundation for regular diagnosis. This emphasizes how important it is to identify and quickly find etiological agents at the farm level; many advance diagnostic methods have been developed for this purpose such as infra-red thermography (IRT), proteomic based diagnosis, sequencing, nanotechnology based diagnosis, specific Immunoassays, sensor for mastitis detection, enzymatic biomarker,specific culture, polymerase chain reaction, micro-RNA based diagnosis, Recombinase polymerase amplification (RPA) and High resolution-melt analysis. Mastitis is multi-etiological complex disease as it is the outcome of interaction of various factors: the host, pathogens and the environment. The disease is characterized by physical, chemical and bacteriological changes in the milk and pathological changes in the glandular tissue of the udder. The global prevalence of clinical mastitis (CM) and subclinical mastitis (SCM) was 15% and 42%, respectively. Buffaloes are thought to be less prone to mastitis compare to cow. Improved milking hygiene, implementation of post-milking teat disinfection and maintenance of milking machinery are all general methods to avoid new cases of mastitis.

Key words: Culture, Epidemiology, Etiology, micro-RNA, Subclinical mastitis.

The name "mastitis" comes from the Greek word "matos," which means "breast" or "udder" and the suffix "itis," which means "inflammation" (Sangam *et al*., 2021; Ibrahim, 2017). Bovine mastitis is an inflammation of the parenchyma of the mammary gland that can cause pathological changes in the glandular tissue as well as physical, chemical and pathogenic bacteria overload in milk (Mellata, 2022). In clinical instances there may be discoloration, a clot, a high number of leucocytes in milk and swelling, pain, heat and edema in the udder (Thakur *et al.,* 2020). Bubaline mastitis is less common and economically significant disease (Johri *et al*., 2023; Jhambh *et al*., 2017). Mastitis in cattle and buffalo is a major global health issue that affects countries such as India, Canada, Germany, the United Kingdom, the Netherlands and the United States of America (Sharun *et al*., 2021). The daily loss of milk from bovine mastitis ranged from 1.0 to 2.5 kg in the first two weeks and the overall loss over the course of the lactation was 110 to 552 kg, depending on the parity and the time of commencement. Since cows will not achieve their peak milk supply throughout the remaining portion of lactation, mastitis also has a long-lasting influence on milk yield (Rajala-Schultz *et al*., 1999). India is the world's largest producer of milk (both cow and buffalo milk together). In India, mastitis causes a 21% decrease in milk production and an estimated Rs. 575 million in economic losses annually (Bardhan *et al*., 2013). Mastitis milk poses a zoonotic risk and is not fit for consumption or sale, which results in significant financial losses. Animals with infected udders are less expensive to buy and place a financial strain on their owners due to medical expenses (Seegers *et al*., 2003). The diagnosis of mastitis is the main requirement for the dairy business to provide clean milk for reasons of economics, public health and animal welfare. For the goal of managing or treating mastitis, early recognition of the condition is crucial for prompt, precise and timely diagnosis. This calls for the use of both traditional and cutting-edge diagnostic procedures.

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Conventional procedures are generally non-specific, but they are also straightforward to use, inexpensive and quickly available in the field. The advanced tests are expensive, necessitate technical expertise and require sophisticated facilities and infrastructure; yet, they are typically accurate and specific for various kinds of mastitis (Chakraborty *et al*., 2019). This review article describes the advance diagnostic methods for udder infection.

Major pathogen causing mastitis

Mastitis may result from any type of physiological harm, pathogenic germs, or chemical irritants that elicit an immunological response (Bramely *et al*., 1996). Bacteria, fungi and yeasts may play a role; but of these, bacteria have by far the largest part (Kibebew, 2017). Pathogenic bacteria and mastitis were found to be associated in 1887; however, the primary pathogens were not recognized until the 1940s. More research on the etiology of bovine mastitis was made possible by the 1960s discovery of its multifactorial nature (Ndlela *et al*., 2016). It has been claimed that around 140 distinct pathogenic species exist (Zeryehun *et al*., 2017). It is thought that the primary cause of mastitis is bacterial intra-mammary infection (IMI). Both Gram-positive and Gram-negative bacteria can cause mastitis (Ashraf and Imran, 2020). Bovine mammary gland infections can be caused by bacteria belonging to three main categories: opportunistic, environmental and contagious pathogens (Hawari and Hassawi, 2008). During the milking process, infectious bacteria that reside on the udder spread from infected to uninfected teats. *Staphylococcus aureus*, *Mycoplasma bovis* and *Streptococcus agalactiae* are the principal ones. Opportunistic infections are able to infiltrate the gland's inner lining due to their potent adhesive qualities. They may result in sporadic bouts of severe mastitis (Fox and Gay, 1993). Environmental pathogens often live in the bedding and housing and they often enter the teat canal when the cow is being milked. Environmental mastitis is primarily caused by *E. coli* (Gunther *et al*., 2011). Mastitis is typically caused by three common streptococcal species: *S. uberis,S. dysgalactiae* and *S. agalactiae*. Other environmental bacteria include *Serratia, Pseudomonas, Proteus* and other Gram-negative bacteria as well as *Klebsiella, Citrobacter* and *Enterobacter spp*, including *E. faecalis* and *E. faecium* (Radostitis *et al*., 2000). Opportunistic pathogens known as coagulase-negative staphylococci (*S. epidermidis, S. simulans, S. saprophyticus* and *S. chromogenes*) infect the lining of the teat or udder surface (dos Santos Nascimento *et al*., 2005). The two most common pathogens worldwide that cause bovine mastitis are coagulase-negative staphylococci and S. aureus (Graber *et al*., 2007). *Candida spp., Cryptococcus neojormans, Saccharomyces spp.* and *Torulopsis spp.* are instances of yeast infections, while *Aspergillus jumigatus*, *Aspergillus nidulans*, *Pichia spp.* and *Trichosporon spp*. are examples of fungal infections. Leptospirosis infections, such as *Leptospira interrogans* serovar *pomona* and particularly *Leptospira interrogans hardjo*, damage the blood vessels in the mammary gland and result in a noticeable irregularity in the milk. Algal infections of mammary gland include *Prototheca trispora* and *Prototheca zopfii*. Although they are not very important, several viruses can also cause mastitis in cattle. Some other bacteria which involve in mastitis are *Citrobacter spp., Enterococcus jaecalis, Enterococcus jaecium, Proteus spp., P. aeruginosa* and *Serratia spp*.; other Gram-negative bacteria include *Nocardia asteroides, Nocardia brasiliensis and Nocardiajarcinica; Histophilus somni; Pasteurella multocida, Pasteurella mannheimia*; *Campylobacter jejuni*; *B. circus*. Anaerobic bacteria have been identified from mastitis instances; these bacteria are typically found in conjunction with other facultative bacteria, such as *Fusobacterium necrophorum, Eubacterium combesii, Prevotella melaninogenica* and *Peptostreptococcus indolicus* (Hamadani *et al*., 2013).

Types of mastitis

Based on the level of inflammation, mastitis is categorized into three classes: clinical, sub-clinical and chronic mastitis (Han, 2020). Visible abnormalities such as a reddened and swollen udder make clinical mastitis clear and easy to recognize; fever milk appears watery and contains flakes and clots (Maros Cobirka, 2020). Acute, sub-acute and per-acute forms of clinical mastitis can be distinguished based on the level of inflammation. In sub-clinical mastitis, there are no outward signs of abnormalities in the udder or milk; yet, as the somatic cell count (SCC) rises, milk production falls (Kibebew, 2017). So the development of a rapid diagnostic test for mastitis is necessary, especially for subclinical mastitis in milk at the earliest stage of infection (Salvador *et al*., 2014). The first pathogenic change observed in subclinical mastitis is the presence of leukocytes and erythrocytes into milk, which increases the permeability of mammary capillaries and triggers an inflammatory response (Tripti *et al*., 2018). Subclinical mastitis was more common in India and Staphylococcus species were more common than other pathogens in mastitis (Krishnamoorthy *et al*., 2021). Additionally, it has been documented that environmental pathogens are more likely to induce the clinical form of mastitis, while infectious causal pathogens are more likely to generate long-lasting subclinical mastitis infections, which are thought to be persistent in a significant number of animals (Sangam *et al*., 2021). When an animal receives insufficient care during the clinical stage of mastitis, chronic mastitis develops.In case of chronic type mastitis, quarters grow hard and antibiotic treatment frequently fails. The structure of milk is often lumpy and the udder swells up extremely red (Sangam *et al*., 2021).

Epidemiology

The global prevalence of clinical mastitis (CM) and subclinical mastitis (SCM) was 15% and 42%, respectively (Krishnamoorthy *et al*., 2021). Sub-clinical mastitis is 15-40 times more common than clinical mastitis

(Ndahetuye *et al*., 2019).Cattle had a greater rate of clinical mastitis (20%) than buffaloes (11%), whereas buffaloes had a higher rate of subclinical mastitis (66%) than cattle (53%). Mastitis prevalence was 85.3% in cows and 78.1% in buffaloes, according to a retrospective study conducted on the antibiogram and prevalence of mastitis in Eastern Haryana's cattle and buffaloes over a six-year period from 2004 to 2009 (Bhanot *et al*., 2012). In addition, both species' month-wise prevalence was higher in warm, muggy months (Ali *et al*., 2021). The economic loss due to subclinical mastitis higher that the acute mastitis in buffaloes (Kashyap *et al*., 2019). The incidence was higher in the hindquarters of buffaloes than cows and both species' right hindquarters were shown to be more vulnerable. The incidence of forequarters was found to be higher in cows than in buffaloes, with the right forequarters being more susceptible (Swami *et al*., 2017). Compared to cows, buffaloes are thought to be less prone to mastitis. However, the viruses that cause the infection can spread swiftly once the buffaloes are infected since buffalo milk is high in nutrients. Tighter teat sphincters in buffaloes help to better prevent pathogen invasion and the mucin-1 (MUC1) gene, which primarily shields the cell surface from environmental pathogens. This is one of the reasons why buffalo mastitis is less common than bovine mastitis (Amin *et al*., 2023; da Rosa *et al*., 2020). Thus, it was discovered that the incidence was higher in the hindquarters of buffaloes than cows and that both species' right hindquarters were more vulnerable (Swami *et al*., 2017). The hind quarters are more vulnerable to environmental and fecal pollution because of their anatomical location, high production capacity and greater pendulousness than the front parts (Kashyap *et al*., 2019; Shukla *et al*., 2016). It was discovered that right side quarters were more vulnerable. Because the animals assume a sitting position on their right side, the pressure on their right side quarters widens the teat canal, allowing pathogenic organisms to enter (Swami *et al*., 2017; Shukla *et al*., 2016). The milkman's initial handling of the animal's right rear quarter while milking may have increased the risk of infection (Fig 1) (Shukla *et al*., 2016).

Risk factors

Mastitis is considered to be a typical example of complex diseases, known to be established as a result of the interactions of three bio-systems namely the causative agent (pathogen), the animal (host) and the environment in which the animal lives.

Pathogen factor

The pathogen factor classified into 2 types based on the bacterial origin-contagious and environmental (Lakew *et al*., 2019). The term "contagious mastitis" describes mastitis that can spread from cow to cow, particularly when milking (Breen, 2017). Environmental infections typically reside in the herd's bedding and housing rather than on the udder and teat skin of cows. The best way to characterize them is as opportunistic pathogens, as they hunt for opportunities to infect (Bradley *et al*., 2012).

Host factor

Consists of animal age, breed, number of parities, stage of lactation, the shape of udders, teat end morphology, teat and udder lesion (Amin *et al*., 2023).

Age

Higher in buffalo aged 7 to 18 yr than in those aged 3 to 6 yr due to the structural changes in the udder and teats and the gradual suppression of the buffalo immune system. Older cows are more susceptible to infections, most probably because of the wider or permanently partially-open teat canal as a result of frequent milking (Kibebew, 2017).

Breed

Relevance of bubaline mastitis is higher in crossbred buffalo than in indigenous breed because crossbred buffaloes can produce more milk (Amin *et al*., 2023).

Stage of lactation

Prevalence of bubaline mastitis in the early lactation stage (14 to 100 days) is higher than in the late lactation stage (more than 200 days) and the mid-lactation stage (100 to 200 days) due to a gradual increase in milk production (Kavitha *et al*., 2009).

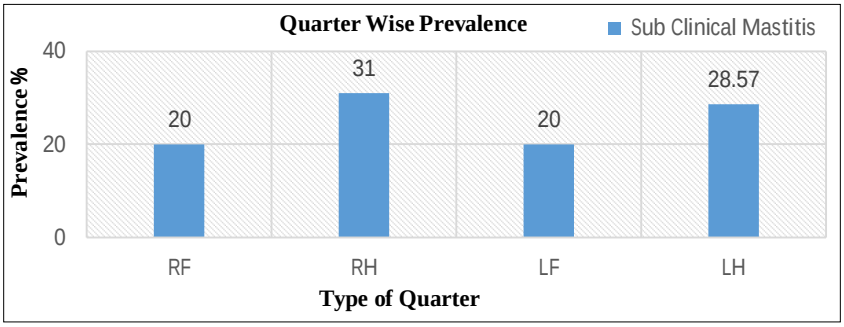


Fig 1: Bar diagram showing quarter wise prevalence of sub-clinical mastitis.

Number of parity

Multiparous cows are more vulnerable to IMI than primiparous cows due to immune-incompetence (Elbayoumy *et al*., 2024; Jingar *et al*., 2014).

Udder structure

The prevalence of bubaline mastitis is higher in buffaloes with bowl- or round-shaped udders than cup shape udders and higher in cylindrical and round teat ends than pointed teat ends (Amin *et al*., 2023).

Enviromental

FactorAnimal welfare and health are significantly impacted by the management techniques used by the herds and the surrounding environment. Mastitis occurrence and severity can be decreased by maintaining a clean, pleasant herd. Mastitis can occur more frequently in cows due to factors such as excessive stocking density, polluted floors, damp bedding, inadequate ventilation and hot, humid weather that encourage the growth of mastitis bacteria and increase cow exposure (Sharun *et al*., 2021).

Advance diagnostic methods

Mastitis is a complex illness that affects dairy cows all over the world. It has a big impact on a country's economy and social cohesion. Therefore, it is imperative to come up with a prompt and accurate solution (Said *et al*., 2022). Advanced testing for mastitis are quantitative, highly specific and sensitive, while conventional tests are typically qualitative with lower specificity and sensitivity (Chakraborty *et al*., 2019; Godden *et al*., 2017). The latest developments in the diagnosis of mastitis are listed in Fig 2.

Proteomic-based diagnosis

By this method pathogens could be accurately, sensitively and quickly diagnosed (Kour *et al*., 2023). The term "proteome" refers to all of the proteins that are present in a cell or tissue at any given time. The goal of proteomics research is to quantify changes in protein abundance throughout the pathological circumstances being studied, as well as to identify the proteins that are present in tissue samples during different physiological stages (Katsafadou *et al*., 2019). Proteomic investigations of milk obtained during bovine mastitis have been conducted using a variety of techniques, including tandem mass spectrometry and liquid chromatography combined with MALDI-TOF MS after two-dimensional gel electrophoresis (2D-GE) (Chakraborty *et al*., 2019). Barreiro *et al*. identified *S. aureus*, *E. coli*, *Strep. agalactiae*, *Strep. dysgalactiae* and *Strep. uberis* from contaminated milk samples with use of MALDI-TOF MS (Kour *et al*., 2023). Early on in subclinical mastitis, milk whey contains protein indicators that can be used as a reliable diagnostic tool to identify mastitis using comparative proteomics (Bian *et al*., 2014).

Sensors for mastitis detection

These diagnostic tools often cause the animal as little stress as possible when detecting mastitis. In large farms, these diagnostic systems are quite useful.Sensor systems are employed in the detection of clinical mastitis in cows (Hogeveen *et al*., 2010).Manual milking has been superseded by machine or automatic milking in large, well-organized dairy farms. The automatic detection of mastitis cases from such a large number of animals requires the use of suitable sensing technology, such as quarter-based milk electrical conductivity (EC) and in-line monitoring of somatic cell count (ISCC) sensing technique (Kamphuis *et al*., 2008).

Enzymatic biomarker

There is release of various enzymes in the milk due to the immune responses of the animals against various infectious. There is a tendency of reduction of the enzymes that deal with synthesis of milk along with increased activity

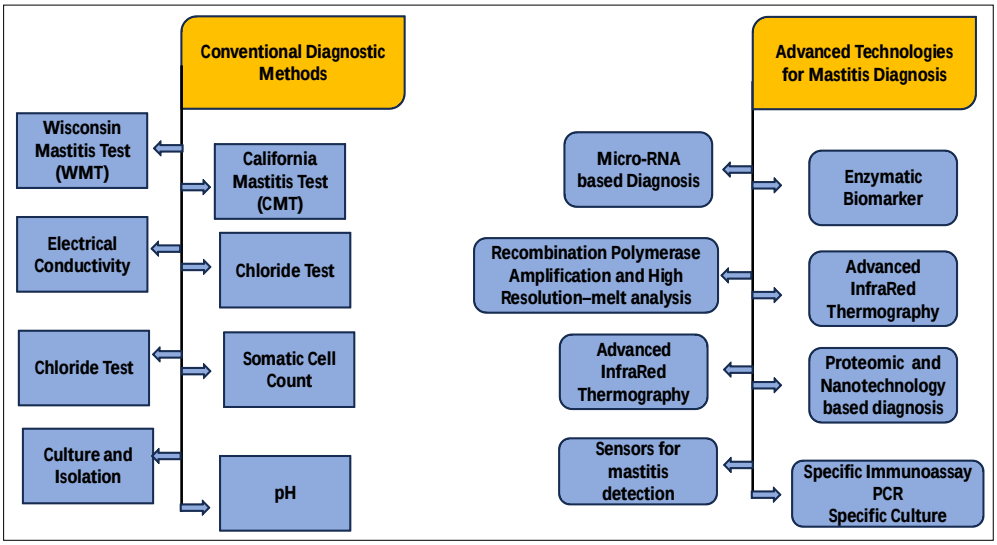


Fig 2: Conventional and advanced diagnostic methods of mastitis.

of the enzymes found in relation to inflammation (Chakraborty *et al*., 2019). Increase in the activities of enzymes that originate from phagocytes such as N-acetyl-D-glucosaminidase (NAGase), milk LDH, ALP, arginase and catalase along with b-glucuronidase (Preethirani *et al*., 2015). With its high sensitivity and specificity and ease of measurement, lactate dehydrogenase (LDH) activity in milk has been demonstrated to be a promising biomarker for subclinical mastitis in buffaloes (Singh *et al*., 2016).

Nanotechnology based diagnosis

Biosensors are devices with a physical nanotransducer (sensor) and bioreceptors specific to the antigen or chemical under study. These sensors detect the presence of specific biological substances via electrical signals (Martins *et al*., 2019). For instance, the development of a real-time mastitis sensing diagnostic based on the identification of acute-phase proteins produced by the liver (such as haptoglobin) was made possible by nanotechnology (Nirala and Shtenberg, 2020). The development of a colorimetric biosensor assay based on magnetic nanoparticles was prompted by the discovery that plasmin's increased proteolysis of casein during mastitis signifies a decline in milk quality. The milk from animals with and without mastitis can be distinguished using this assay. A direct indicator of plasmin proteolytic activity is the biosensor's heightened golden color, which is caused by plasmin bound to magnetic nanoparticles being present as a monolayer across its surface. The *in vitro* levels of plasmin (1 ng/ml) in the milk samples can be detected by this biosensor with extreme sensitivity (Chinnappan *et al*., 2017).

Infra-red thermography (IRT)

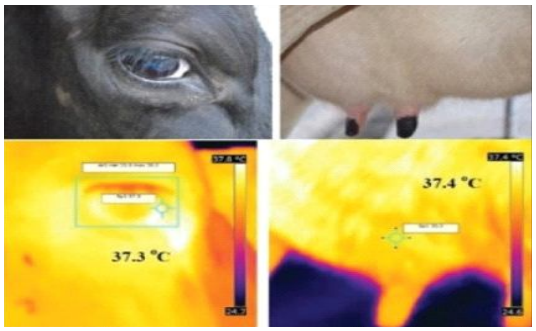
The ability of both CMT and infrared thermography (IRT) to differentiate between clinical and subclinical mastitis makes them practically identical. A novel method for early mastitis diagnosis that is practical, portable and may be applied on-site is infrared thermography. It is predicated on the temperature differential between diseased and healthy udders. The degree of udder infection is ascertained by analyzing the heat images that thermal cameras capture. Warmest region appears as white or red, whereas the coolest region appears as blue or black (Fig 3 and Fig 4) (Sathiyabarathi *et al*., 2016).

Specific immunoassays

Immunoassays have been used to identify milk amyloid A, acute phase proteins (haptoglobin) Hp, Cytokines, such as tumor necrosis factors and interleukins. which rise in milk during inflammation, in the diagnosis of bovine mastitis (Al-Rasheed *et al*., 2022; Duarte *et al*., 2015). A number of research provide sophisticated instruments for the earliest diagnosis, such as an indirect ELISA for the detection of antibodies against *Streptococcus agalactiae* rAP1-BP-AP2 proteins and rSip-PGK-FbsA fusion protein and a biomarker-based Liquid Phase-Blocking ELISA for subclinical mastitis (Kour *et al*., 2023; Bu *et al*., 2017). For the purpose of identifying and evaluating the cathelicidin proteins found in the milk of buffalo, researchers have created and standardized ELISA. When mastitis occurs, the milk secretes cathelicidin, which are tiny proteins with antibacterial properties linked to innate immunity (Puggioni *et al*., 2020; Chakraborty *et al*., 2019).

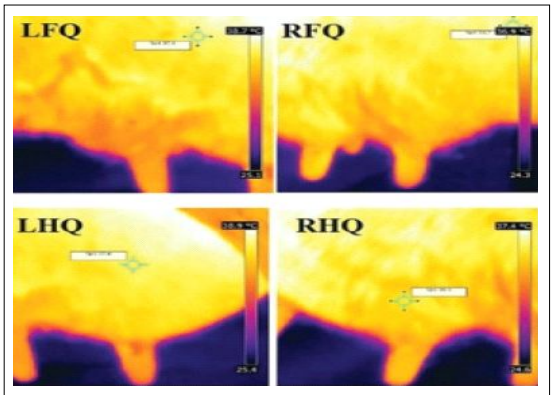
Specific culture

Microbiological/bacteriological culture is utmost required for microbial or bacteriological etiology of (subclinical) mastitis (Abdelmegid *et al*., 2018). However, in 10-40% of cases with clinical mastitis at the quarter level, a bacteriological investigation of milk samples reveals no growth. Such a situation could arise for a number of reasons, such as the presence of very few organisms or the possibility that samples contain diseases like Mycoplasma spp. that need to be cultured using specialized media and techniques (Chakraborty *et al*., 2019). Bacterial culture techniques are much less sensitive (32.2%) than PCR assays (70.6%) in identifying the pathogens responsible for mastitis (Vidic *et al*., 2018).



The image adopted from (Sathiyabarathi *et al*., 2016).

Fig 3: Infrared thermogram and visual image of eye and udder surface.



RHQ- Right hind quarter, LHQ- Left hind quarter, LFQ- Left front quarter, RFQ- Right front quarter. This image adopted from (Sathiyabarathi *et al*., 2016).

Fig 4: View from lateral side of infrared thermogram of udder quarters.

Polymerase chain reaction (PCR)

PCR is culture-independent methods for identifying bacterial pathogens in milk. PCR is faster (result within 4 hours) and more sensitive compare with traditional culture (Adkins and Middleton, 2018). It involves DNA extraction and repeated cycles of denaturation, amplification and replication, in which segments of deoxyribonucleic acid (DNA) are continuously multiplied.A particular type PCR, known as multiplex PCR, can amplify multiple sequences of DNA in one reaction (Journal *et al*., 2019). RT-PCR uses reverse transcription to produce a DNA template from an RNA source that can then be amplified (Lu *et al*., 2014). Reverse transcription (RT)-PCR tests have been developed to both identify and measure pathogens associated with mastitis in milk. The application of these molecular techniques has grown in popularity for the purpose of differentiating bacterial strains within a species (Shome *et al*., 2011). RT-PCR allows for instantaneous detection, can be quantitative (qRT-PCR) and can even identify tiny folds (2-folds) also (Keane *et al*., 2013). However, conventional PCR is non-quantitative and provides a basic identification of the organism based on the amplification of genetic material or DNA at the end point. It also has low resolution because it can only detect at higher folds (10 or more) (Duarte *et al*. 2015). PCR is asensitive (76.9-100%) and specific (63.3-98.7%) technique for diagnosing mastitis (Paramasivam *et al*., 2023). PCR has been applied to both single and large-scale milk samples and for clinical and subclinical mastitis (Syring *et al*., 2012).

Microbial typing/finger printing/sequencing/characterization

Sequencing has emerged as a key diagnostic technique for mastitis that allows bacteria to be identified by species, subspecies and strain level (Chakraborty *et al*., 2019). Bovine isolates from human isolates must be distinguished from each other due to the zoonotic significance of the isolates' subspecies differentiation (El-Sayed *et al*., 2017). For the purpose of genotyping, a variety of molecular techniques have been used, including ribotyping (Choudhary, 2018), pulsed-field gel electrophoresis (PFGE) (Pumipuntu *et al*., 2019) amplified fragment length polymorphism (AFLP) (Mohajeri *et al*., 2016), random amplified polymorphic DNA (RAPD) (Tomazi *et al*., 2018) and multilocus sequence typing (MLST) (Pumipuntu *et al*., 2019). DNA is utilized in these techniques after being digested with restriction enzymes, amplified using PCR, sequence analysis, or by combining all of these techniques (Choudhary *et al*., 2018). Multi-locus variable number tandem repeat analysis (MLVA) and ribotyping, as well as study of transfer DNA intergenic spacer length polymorphism, can be carried out at the strain and species levels respectively (Pinho *et al*., 2012). *S. aureus* isolates from bovine mammary secretions can be successfully subjected to genetic analysis using PFGE in conjunction with binary interspace (IS) typing. It has been discovered that binary IS typing is a reliable technique that is easy to use and has a lot of potential to develop into a potent instrument for characterizing bacterial strains (Zadoks *et al*., 2001). PFGE is used to separate large DNA molecules. Better size resolution can be achieved using PFGE as opposed to agar gel electrophoresis. It has been determined that the PFGE procedure is quite appropriate for studying the *Streptococci* that cause mastitis in cows (Santos-Sanches *et al*., 2015). Both PFGE and MLST have been used to genotype and characterize different strains of *S. aureus* that cause mastitis in cows (Pumipuntu *et al*., 2019). For the purpose of differentiating *S. uberis* isolates from milk, MLST was developed. By using this specific technique, two new clonal complexes have been identified: sequence type (ST-86) and ST-143 (Tomita *et al*., 2008).

Recombinase polymerase amplification (RPA)

It is now possible to amplify specific DNA sequences in a way that differs from PCR thanks to a recently developed and promising technology. The introduction of two additional proteins in addition to the polymerase-recombinase proteins and single-strand binding proteins-is essential to the novel technique known as recombinase polymerase amplification (RPA). Under isothermal conditions, the reaction is conducted. It is possible to utilize the used cycler as a portable device because it is noticeably smaller than PCR thermocyclers. There exist numerous RPA variations, including multiplex RPA, on-chip RPA and reverse transcription recombinase polymerase amplification (RT RPA) (Daher *et al*., 2015; Kersting *et al*., 2014).

Micro RNA (miRNA) based diagnosis

MicroRNAs are naturally occurring, tiny, non-coding RNAs that are produced by different kinds of cells and released into extracellular environments and biofluids, where they control a range of biological activities within cells (Srikok *et al*., 2020). Changes in the expression of different microRNAs (MIR146A, MIR155, MIR184, MIR24-3 p, MIR148, MIR486 and LET7A-5p) were seen in the in vitro challenge investigations including *E. coli* lipopolysaccharides and *Staphylococcus aureus* enterotoxin B. MIR184, MIR24-3 p, MIR148, MIR486 and LET7A-5p were identified by Jin *et al*. (2014) as distinct microRNAs linked to *E. coli* intramammary infection (IMI) (Jin *et al*., 2014).

High resolution-melt analysis

Using traditional culture-based approaches compromises the accuracy of mastitis pathogen detection. Here, we describe a new, quick assay that uses high-resolution melt analysis (HRMA) of 16S rDNA sequences to screen for the speciation of bacterial mastitis pathogens. A 290 bp amplicon was obtained from the real-time PCR amplification of the 16S rRNA gene segment, which spans the variable regions V5 and V6. Initially, a library comprising the melt curves of nine prevalent pathogens linked to bovine mastitis was created. Three of the isolates-*Arcanobacterium pyogenes*, *Corynebacteriumbovis* and *Streptococcus*

dysgalactiae-were field isolates related to bovine mastitis, while the remaining six-*Escherichia coli*, *Streptococcus uberis*, *Klebsiellapneumoniae*, *Staphylococcus aureus* and *Mycoplasma bovis*-were type strains. Three of the type strains were identified from illnesses in cows, whereas the other four, *S. aureus, K. pneumoniae, S. agalactiae* and *E. coli*, were discovered to be of human origin.Second, ten bovine mastitis field isolates of each pathogen were compared to the melt curves and associated amplicon sequences of *A. pyogenes*, *E. coli, S. agalactiae*, *S. dysgalactiae*, *K. pneumoniae*, *S. uberis* and *S. aureus*. The selection of a collection of bovine strains for these pathogens to be utilized as reference strains in the HRMA was deemed required due to the notable discrepancies in melt curves and sequencing between human and bovine isolates of *E. coli* and *K. pneumoniae*. Through analysis of the melt curves of 60 bacterial cultures recovered from mastitis milk samples, three interpreters verified the HRMA. The culture and sequencing results of the isolates were concealed from the three test interpreters. A 95% validation test accuracy was achieved overall because the variability in the *S. uberis* PCR amplicons made it challenging to identify the *streptococci.* According to this study, differentiating between clinically significant bacterial mastitis pathogens can be accomplished quickly, effectively and affordably using broad-range real-time PCR in conjunction with HRMA.

CONCLUSION

Mastitis impairs animal wellbeing and results in financial and productivity losses due to declining milk quality, decreased output, higher treatment costs and decreased production performance. Subclinical mastitis can lower milk quality to the point that it cannot be seen visibly but still affects the product, it is more economically significant than clinical mastitis. For the diagnosis of mastitis, a number of reliable and affordable conventional diagnostic methods are available; however, they are not very sensitive or specific. Since they can't produce results quickly, they can't be used widely in the dairy production industry as it stands today. Current advancement in mastitis diagnosis has greatly enhanced the capacity to identify and treat the condition with greater efficiency. Somatic cell count (SCC) has been improved by incorporating modern technologies like PCR-based pathogen identification, proteomics, biosensors and advanced imaging techniques. These advancements facilitate the identification of pathogens sooner and with greater accuracy, which leads to more focused treatments and a decrease in the unnecessary use of antibiotics. Additionally, quick point-of-care tests have made on-farm detection more efficient, enhancing herd health, milk quality and productivity in dairy farming. The convergence of data analytics and digital tools enhances proactive management, facilitating improved prevention strategies.

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Disclaimers

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Conflict of interest

The authors declare there is no possible conflict of interest.

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