



# Diagnosis of bovine mastitis: from laboratory to farm

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Received: 9 February 2018 / Accepted: 30 May 2018 / Published online: 8 June 2018  
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## Abstract

Accurate diagnosis of disease is the major step between the cause and cure of disease. An economical, reliable, and rapid diagnostic tool is fundamental for the management of udder health. The earlier the disease is identified, the less will be the damage; keeping this in mind, many efforts are being made to develop reliable diagnostic tools for use on farm. However, traditional gold standard methods including somatic cell count and microbial culturing are still in use. They are partially being replaced with polymerase chain reaction and sequencing-based tests. Nanotechnology and protein-based tests have also gained lot of attention and some of them are potential candidate of future diagnostic tests for bovine mastitis. Research laboratories are struggling to develop simple, economical, and user-friendly biosensor-based methods that can be performed on farm for rapid diagnosis. The combination of both genomic and proteomic approaches, together with further involvement of nanotheranostic technologies and other sensors, will assist in the quest of better mastitis diagnostic tools.

**Keywords** Bovine mastitis · Diagnosis · Traditional methods · Modern diagnostic tools

## Introduction

Inflammation in the mammary gland results in decreased milk production, veterinary care costs, and culling losses. For better control and treatment strategies, identification of causative pathogen is critically important. The traditional methods rely on microbial culturing and biochemical tests. These methods are time consuming and allow the detection of only viable bacteria. Due to this reason, false negative results are obtained which lead to greater damage and production loss. Molecular detection-based methods have the potential to detect the pathogenic organisms from milk samples that otherwise would be false negatives (Taponen et al. 2009).

## Diagnosis of bovine mastitis: present and future

Accurate diagnosis of disease is the major step between the cause and cure of disease. An economical, reliable, and

rapid diagnostic tool is fundamental for the management of udder health (Mahmmod 2013). The earlier the disease is identified the less will be the damage, and with this in mind, many efforts are being made to develop the reliable diagnostic tools. Mastitis is an inflammation of mammary gland as a result of invasion by some pathogen, allergy, or physical trauma (Zhao et al. 2008). Bovine mastitis is one of the most common diseases of the dairy industry due to huge economic losses in terms of reduction in milk production, treatment costs, and culling (Viguier et al. 2009). Depending on the severity of infection, there are two types of mastitis, namely clinical and subclinical. In clinical mastitis, physiological symptoms appear and changes occur in the milk appearance and udder. No visible symptoms are observed in subclinical mastitis.

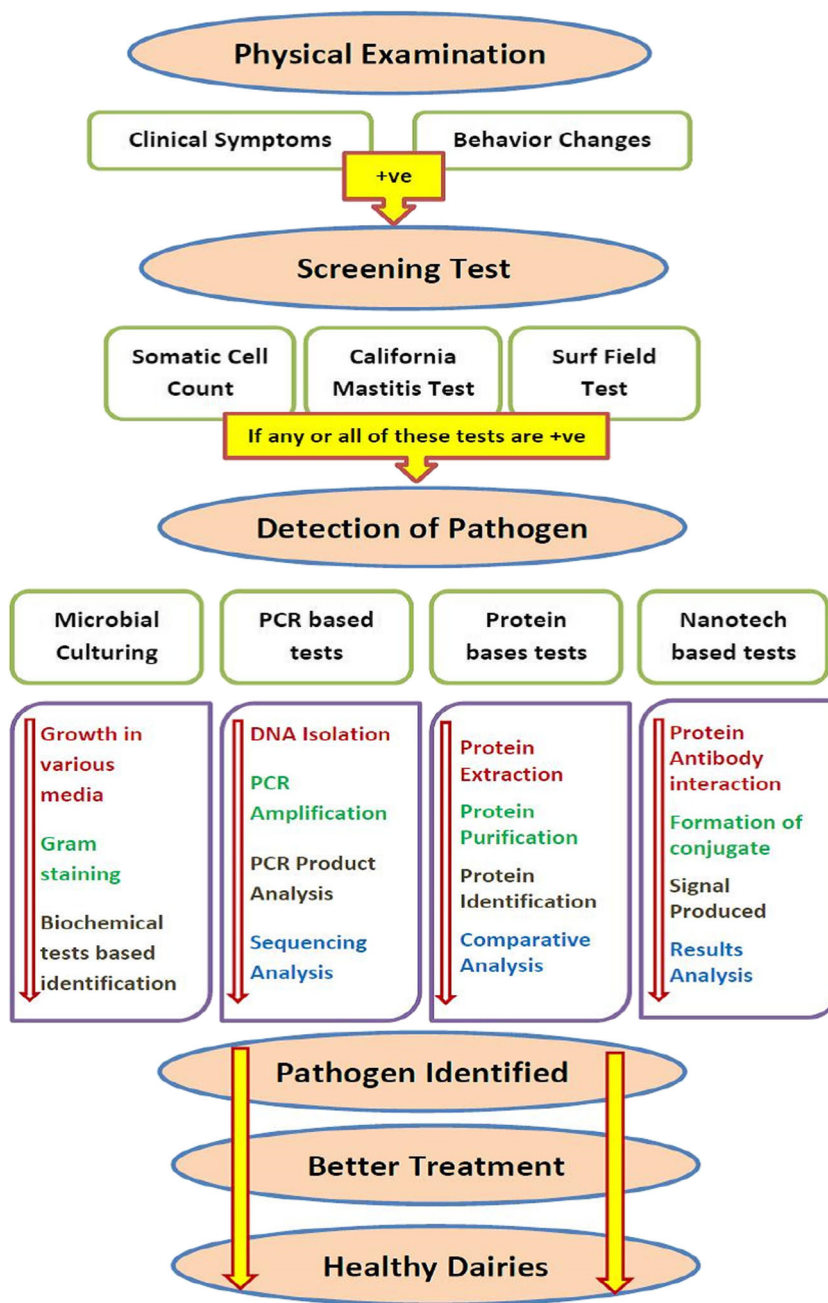
In simple terms, there are two stages of disease diagnosis: first is an indicator of disease status if it is present or not, and in the second stage, the causative agent is detected. A brief outline of diagnosis of bovine mastitis is shown in Fig. 1. Disease status is indicated by the visible appearance of the udder and milk in the case of clinical mastitis.

For subclinical mastitis, on-farm screening tests are used traditionally, such as somatic cell count (SCC), California mastitis test, and Surf field mastitis test. None of the abovementioned methods indicate causative agent and quantitative results for level of severity (Viguier et al. 2009). SCC

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**Fig. 1** Diagnosis of bovine mastitis: an overview



is considered as a universal indicator for mastitis detection and is widely used for determining milk quality in individual quarters and bulk tanks. It has few disadvantages such as cost of test and requirement of trained staff (Rasmussen 2001).

Inflammation in mammary gland results in decreased milk production, veterinary care costs, and culling losses. Despite considerable knowledge about mastitis and its etiology, this disease is still prevalent in many dairy herds; it remains most difficult to eradicate or control, and it has a great negative financial impact on the dairy industry worldwide. These losses are caused by several factors such as a decrease in the total milk output with marked compositional changes in the milk (a

reduction in the quality and industrial usability), cost of treatments, labour costs, and increasing the chance of premature culling of cows (Halasa et al. 2007). Decreased milk production is often due to the direct effect of pathogen or damage caused by the host immune response. Mastitis is very complicated in terms of etiology, degree of losses, and treatment strategy (Detilleux et al. 2015).

Mastitis not only affects the health of animals and results in reduced milk production but it also has a negative impact on quality of milk. Subclinical mastitic milk samples have decreased milk fat, protein, lactose, potassium, calcium, magnesium, phosphorous, iron, and zinc and increased pH, electrical

conductivity, malondialdehyde, leukocytes, and neutrophil count (Qayyum et al. 2016).

## Diagnostic methods

One key component of better control of this disease is identification of the causative bacterial agent during udder infections in cows. Given the diversity of pathogens that must be identified, mastitis is a complex disease. Development of a rapid and efficient bacterial species identification tool is thus necessary. Early detection of mastitis and identification of the causative agent is crucial for control and treatment. The initial methods used for detection are (1) estimation of SCC, an indication of inflammation; (2) measurement of biomarkers associated with the onset of the disease (e.g., the enzymes *N*-acetyl- $\beta$ -D-glucosaminidase and lactate dehydrogenase); and (3) identification of the causative microorganisms, which often involves culturing methods. These methods have their limitations and there is a need for new rapid, sensitive, and reliable assays. Significant advances in the identification of nucleic acid markers and other novel biomarkers and the development of sensor-based platforms have taken place (Viguier et al. 2009).

## Traditional methods

Mastitis diagnosis begins with the observation of any apparent change in the mammary gland or milk followed by other clinical signs such as fever, weakness, or loss of appetite. Staff who manage the dairy herd have an important role to play in this initial screening and visual observation of symptoms (Lam et al. 1993). Well-trained and vigilant staff can help in better control and management of mastitis by not ignoring even a minor change in the udder tissue or a smallest clot in milk. The abovementioned symptoms are an indication of intramammary infections and, if taken into account, can help initiate early treatment and control the spread of disease (Hulsen et al. 2008).

The abovementioned physiological symptoms appear at the clinical stage of mastitis. It is complex disease not only in terms of etiology but it shows many symptoms which vary depending on the nutritional status of animal, pathogenic strain involved, and other factors. SCC is often considered as most widely used biomarker for detection of bovine mastitis and is linked with infection status in terms of severity and stage (Harmon 1994). SCC levels can also increase slightly by other stress factors which are not related with mastitis. Currently, the threshold of 200,000 cells/mL is used to indicate the status of disease if it is present or not (Schukken et al. 2003).

Various methods are used for determination of SCC which include direct microscopic counting using methylene blue

staining, coulter counting, and fluoro-optic electronic cell counting by disk or flow cytometry. Direct microscopic counting is the reference method but has many limitations. It is time consuming and skilled labor is required and it is difficult to differentiate between cells and cytoplasmic particles (Moon et al. 2007). Electronic particle counting is performed with a Coulter counter. It involves the addition of formaldehyde prior to performing the test to fix the somatic cells and cell lysis treatment to remove fat cells (Miller et al. 1986). The indirect methods for SCC determination are fossomatic, somacount, and somascope based on disk or flow cytometry. These methods are equally good for fresh as well as preserved milk and no pre-treatment is required. Flow cytometry combined with fluorescence staining gives a better option as a rapid reliable and affordable test (Gunasekera et al. 2003).

California mastitis test (CMT) and Surf field mastitis test (SFMT) are very simple and can be performed on farm for SCC estimation (Schalm and Noorlander 1957). The reagent used in CMT is sodium alkyl aryl sulfonate, and SFMT requires the commonly used detergent “Surf” so it is cheaper and conveniently available in low-income countries. However, results obtained are comparable to each other. It is based on the principle that nucleic acids and other cell constituents are released in the presence of high SCC and a gel is formed which can be easily detected (Sargeant et al. 2001; Muhammad et al. 2010). The few drawbacks linked with these tests include false positive and false negative results and none of these tests give any numerical values of SCC. Further, these tests do not identify the pathogen involved and severity of infection but only give mastitis positive or negative results (Viguier et al. 2009). Among cow side indicators, the firmness of the udder can also serve as an indicator of bovine mastitis. Firmness of infected and normal udder before and after milking was checked and considerable increase in udder firmness of infected udder after milking was observed. Further studies on udder firmness and its relationship with disease may lead to its application as a predictive indicator of mastitis (Rees et al. 2017).

## Detection of pathogens

All of the abovementioned methods are indicative of disease and some of them provide information about the stage or severity of mastitis. However, none of them can identify the causative agent. Many advantages are related with the early and accurate detection of the pathogen involved. These include the choice of treatment method, antibiotic selection, and better management strategies to control the spread of disease in the case of a contagious organism. Researchers from various disciplines including dairy scientists, microbiologists, molecular biologists, biochemists, and nano-technologists are

struggling to create a suitable, simple, accurate, and cheap test for detection of mastitic pathogens.

## Microbial culturing methods

Pathogens involved in various veterinary diseases are usually identified by microbial culturing or antibody-based detection methods (Schmitt and Henderson 2005). Detection of the causative organism of mastitis has many advantages in terms of selective use of antibiotics, reduced risk of spread of infection in the case of contagious pathogen, and culling decisions. Bacterial culturing-based detection of pathogens is still considered as a gold standard despite many discrepancies and inconvenience (Hogan et al. 1999). The major discrepancies associated with culturing method are false negative results, as well as being a time-consuming and labor-intensive activity. In samples containing more than one pathogen, one bacterial species may suppress the growth of other species and many bacterial species require a long period of time for growth, such as *Mycoplasma* spp. Results mainly rely on interpretation of phenotypic properties of bacterial culture, so considerable variability is seen. It has been reported already that microbial culturing of mastitis milk samples gave 27–50% false negative results (Makovec and Ruegg 2003; Barrett et al. 2005; Bradley et al. 2007; Koivula et al. 2007; Olde Riekerink et al. 2008).

Despite the abovementioned facts, efforts are still being made to create a better diagnostic assay on the basis of microbial culturing. Various microbial culturing-based kits (e.g., VetoRapid and Vétoquinol) were designed to serve the purpose. The specificity and sensitivity of these on-farm culturing kits were compared with the routine culturing method. These kits offered economic benefits by rapid detection of gram positive clinical mastitis pathogens. These reported results also showed misidentification of certain pathogens in some cases (Viora et al. 2014). On-farm culture systems were devised for early treatment decisions. They are based on the use of selective media for differentiation between various groups of mastitis pathogens. Minnesota Easy Culture System II Bi-Plate and Tri-Plate were evaluated as on-farm culture systems. Results indicated higher specificity but lower sensitivity. These methods were more reliable for broad classification of infections but not very promising for closely related species identification (Royster et al. 2014). These on-farm culture systems did not serve as the replacement of laboratory microbial culturing; however, acceptable results were obtained to make quick treatment decision for mastitis cases.

## PCR-based methods

Use of molecular techniques in veterinary diagnostics is not a new strategy. These methods have the potential to detect the

pathogen with higher sensitivity and specificity. The advent of PCR technology along with its various extensions such as multiplex PCR, real-time PCR, and LAMP has improved the rapidity and sensitivity of diagnosis. These DNA-based diagnostic methods have greatly aided in better management of dairy farms. Nucleic acid-based detection relies on the genomic sequences of various pathogens (Lui et al. 2009).

Certain mastitis pathogens do not grow in bacterial culture or are difficult to detect due to their slow growth rate. PCR assays are quite popular in dealing with such issues by rapidly detecting the pathogen with high specificity. One such study was conducted by Boonyayatra et al. (2012) to detect three species of *Mycoplasma* by real-time PCR assay.

PCR electrospray ionization mass spectrometry has been used as a diagnostic tool for bovine mastitis pathogens from milk samples. It can detect not only bacterial species but yeasts, molds, parasites, and viruses can also be used in mixed culture. It has high specificity and sensitivity but is quite costly for use as a routine diagnostic tool (Perreten et al. 2013).

DNA sequencing is usually preferred for identification of bacteria. 16S rRNA sequencing is used largely for identification of closely related bacterial species (Lange et al. 2015). Various techniques are combined in different studies to obtain the best results. One such example is combining real-time PCR with high resolution melting analysis for detection of mastitis pathogens. This method can also serve as an alternative to traditional diagnostic methods and offer a number of benefits including low cost and rapid results (Ajitkumar et al. 2012).

The less common, unviable, slow growing, and phenotypically unique pathogens are always difficult to detect by routine diagnostic methods. PCR and DNA sequencing have provided favorable results that could lead to early treatment and better control strategies for infectious diseases. 16S rRNA sequencing can identify novel bacterial species in various diseases (Woo et al. 2008). Various attempts have been made for rapid identification of a mastitis pathogens by PCR using samples collected on filter paper disks. There are many benefits associated with such kind of methods but not all the pathogens can be recovered from filter paper by this technique. *Streptococcus agalactiae* was detected by this method and the samples can be stored at room temperature for 4 weeks (Jiusheng et al. 2008).

PCR is more sensitive, rapid, and reliable as compared to the bacterial culturing method for detection of various mastitis pathogens (Cantekin et al. 2015). A multiplex PCR assay was designed for the detection of four common mastitis pathogens in a single test. Multiplex PCR assay has many advantages over the traditional techniques in terms of ease, sensitivity, and specificity (Charaya et al. 2015). A recently developed novel multiplex PCR assay for detection of nine important mastitic pathogens in a single reaction was reported. Results were evaluated on milk samples and compared with bacterial culturing and 16S rRNA sequencing by latent class analysis. However,



the newly developed assay proved best among three (Ashraf et al. 2017). A multiplex real-time PCR kit PathoProof™ is available commercially for detection of 11 mastitis-causing pathogens. It has been shown to have higher sensitivity and specificity and is quite rapid when compared with microbial culturing (Koskinen et al. 2009).

Fluorescent in situ hybridization (FISH) is another culture-independent method tested for detection of mastitis pathogens. It is less time consuming and reliable but is not commonly used due to certain limitations such as a pre-treatment step is required to obtain the results and high detection limit (Gey et al. 2013).

Various studies have focused on the development of multiplex PCR assay for detection of various common mastitis pathogens (Pradhan et al. 2011; Riffon et al. 2001). Genus- and species-specific multiplex PCR for detection of *Enterococci* was developed by Jackson et al. (2004). It showed a high percentage of agreement when compared to other available methods for identification of the target species. There is a need of a perfect gold standard test for detection of bovine mastitis pathogens, which is rapid and accurate, specific, and sensitive and can be easily performed. Various studies focused on the development of a multiplex real-time PCR assay for detection of major mastitis pathogens, and this is showing a high potential and future prospects due to high sensitivity and specificity (Gillespie and Oliver 2005; Paradis et al. 2012).

Loop-mediated isothermal amplification (LAMP) is another exciting technology that was first presented by Notomi et al. (2000). In the last few years, it has gained much popularity in the field of diagnostics. It is quite a rapid, economical, sensitive, and specific method that has the potential to be used as a field test with few modifications (Lee 2017). It is based on the strand displacement activity of DNA at isothermal conditions in the presence of *Bst* polymerase. It produces dumbbell-shaped structures which serve as template for the further amplification. As a result, stem loop DNA of varied lengths are produced which give a ladder-like pattern when visualized on agarose gel (Notomi et al. 2000; Tomita et al. 2008). Results can be directly visualized by adding a dye such as SYBR green: a change in the color due to presence of magnesium pyrophosphate, a LAMP byproduct, indicates the sample is positive (Parida et al. 2008).

Various studies are focused on the development of a LAMP assay for the detection of mastitis pathogens. For the detection of major mastitis pathogens such as *Staphylococcus aureus*, *S. agalactiae*, and *Streptococcus uberis* from bovine mastitic milk, a LAMP test was developed by Tie et al. (2012), Bosward et al. (2016), and Cornelissen et al. (2016), respectively. The centrifugal LAMP microdevice has been designed and tested for monoplex identification of mastitis pathogens. It has a calcein-mediated calorimetric wireless detection system which can effectively detect pathogens in 65 min (Sayad et al. 2018). Researchers are currently addressing the

limitations of LAMP which is mainly low sensitivity. Its use in combination with lateral flow assay and lyophilized and electric (e) LAMP has a lot of potential to serve as a field test for diagnosis of various diseases and for sex determination on site (Centeno-Cuadros et al. 2017).

## Probe-based assay/Nanoparticle-based assay

Nanotechnology-based diagnosis of infectious diseases is gaining lot of attention from last few years. Nanotechnology-based biosensors introduced the concept of having a laboratory “on a chip” and can lead to rapid and accurate detection of pathogens (Driskell and Tripp 2009). Nanoparticles together with proteomics can be a future candidate for rapid diagnosis of bovine mastitis pathogen. A study was conducted in which specific protein corona was formed when novel magnetic nanoparticles were added to biological fluid. This can further help in minimizing proteome complexity by analyzing protein corona on nanoparticles (Miotto et al. 2016). Microarray diagnostics in three-dimensional nitrocellulose membranes have been used for diagnosis of mastitis, a biochip used in this study has an inkjet printer, and a set of tag-specific antibodies immobilized on nitrocellulose membrane-coated slides. A conjugate for secondary signal was prepared using black carbon nanoparticles and a fusion protein. The blackness of the spots can be determined by flatbed scanning; however, it can be easily read by the naked eye. This biochip-based assay has the ability to detect four mastitic pathogens in less than 3 h (Mujawar et al. 2013). An oligonucleotide probe-based biochip was developed which has the ability to specifically capture the target bacteria and the pattern obtained on the chip is readable. It has been tested for its specificity and efficacy for detection of seven important bacterial mastitis pathogens. The associated drawbacks of such a biochip are the requirement of a well-equipped molecular biology laboratory and trained staff, but this is not affordable for routine use by dairy farmers (Lee et al. 2008).

## Protein-based diagnostics

The advancements in the field of proteomics have also led to rapid and accurate diagnosis of bovine mastitis. It is commonly used to identify and quantify the proteins expressed in any biological sample and this may lead to a better understanding of their function and involvement in that particular state. It relies on the principle that any change in proteins or proteomic make up of the organism or biological fluid is an indication of diseased or abnormal state. Two-dimensional gel electrophoresis and mass spectroscopy are the most commonly used methods (Lippolis and Reinhardt 2005; Smolenski et al. 2007). Comparative proteomic analysis of mastitic and healthy

milk samples indicate suitable biomarkers and also gives a better understanding of underlying pathological mechanism of disease. Together with two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry analysis was used to analyze the differences in protein, which was further confirmed by obtaining mRNA expression of the target gene by quantitative real-time PCR. Bian et al. (2014) concluded from one such study that these biomarkers will lead to better diagnosis of mastitis. They can serve as a potential candidate to accurately discriminate between healthy and diseased states and also provide some knowledge about the causative agent (Mansor et al. 2013).

Certain polypeptides are released during inflammation, which can serve as a specific biomarker. Cathelicidins, a type of inflammatory proteins, are released in mammary glands before the onset or other physical symptoms. ELISA-based detection of cathelicidins was evaluated for its accuracy and specificity and results were compared with samples with high SCC: a high degree of association was found (Addis et al. 2016). Some studies are planned to broadly identify the group of pathogens for the quick and accurate treatment. One such approach was based on flow cytometry which can distinguish between gram positive and gram negative bacteria. This method has an ability to quantify bacteria in bulk milk tanks, but a few drawbacks including a low limit of detection, cross-contamination, and background noise remain a big hurdle for its common use at farm level (Langerhuus et al. 2012). Capillary electrophoresis and mass spectroscopic studies on milk from healthy cows and mastitic cows, caused by both gram positive and negative bacteria, were performed to identify biomarkers. The biomarkers identified have the potential to be used for diagnosis of mastitis by a gel-free method (Mansor et al. 2013). In a recently reported study, a label-free quantification method was used to determine relative changes in protein expression in mastitic milk caused by *S. uberis*. It has proved to be more advantageous and provide more comprehensive information on host response to bovine mastitis as compared to previously reported studies (Thomas et al. 2016).

Automated milking systems are increasingly being adopted as a field test for diagnosis of mastitis, and measurement of protease activity is one such indicator. Koop et al. (2015) demonstrated a method using fluorogenics protease substrate for rapid and low cost detection. If not all, most of the proteomic studies are conducted using two-dimensional gel electrophoresis, further confirmed by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF)/MS and liquid chromatography (Boehmer 2011). These technological advances resulted in the evaluation of not only an experimentally induced mastitis protein profile but also in naturally occurring infection (Smolenski et al. 2007). Werner et al. (2012) performed diagnostics using MALDI-TOF MS for identification of *Enterococcus* species. *S. aureus* and staphylococci CNS strains, largely involved in causing bovine

mastitis worldwide. Mass spectral identification of these common pathogens was performed by MALDI Biotyper and very promising results were obtained as compared to other available methods (Elbehiry et al. 2016).

## Future of mastitis diagnostics

An ideal diagnostic test should have some features as mentioned in Fig. 2. Inflammatory response is associated with the virulence of causative agent of mastitis, as proven by artificially induced mastitis in mouse models and monitoring various factors involved. However, to find the relationship of these pro-inflammatory factors with mastitis requires further studies (Johnzon et al. 2016). The Fourier-transform infrared (FTIR) spectroscopic technique which is based on DNA fingerprinting of the whole organism can differentiate between closely related species. A study based on this method demonstrated that mastitic pathogens could be accurately detected at low cost, once the system is established and installed (Schabauer et al. 2014).

With all these substantial technological advances in the field of diagnosis, we cannot ignore the importance of monitoring behaviour changes at or before onset of mastitis. A very comprehensive study concluded that if animals are closely observed they show remarkable changes in terms of feed intake and overall activity. These sickness signs are evident few days before clinical symptoms appear, so it can serve as an indicator (Sepúlveda-Varas et al. 2016). For this purpose, an automated health monitoring system was developed that can record the rumination and physical activity. It proved effective when applied in conjunction with other traditional diagnostic

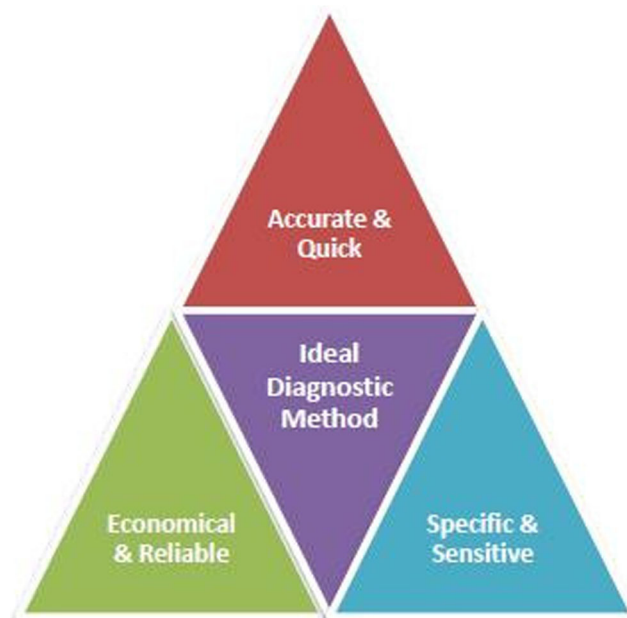


Fig. 2 Characteristics of ideal diagnostic test

methods; however, further progress in the management area is required to maximize the benefits (Stangafferro et al. 2016).

In the quest for simple and accurate detection indicators, a study was performed to monitor udder temperature by infrared thermograph, and it was demonstrated that subclinical cases have a higher udder temperature as compared to healthy ones (Martins et al. 2013). Physical monitoring is often laborious with many hurdles. To circumvent the problems associated with it, animal-mounted sensors linked with an intelligent surveillance system were developed. It is planned to detect the disease a few days before the onset of disease; however, further advancement is required to routinely use it with high specificity and sensitivity (Yazdanbakhsh et al. 2017). Integration of alternate current electrokinetics with biosensing will also help to achieve high sensitivity and will save time (Zhang et al. 2015).

The on-farm culture system Accumast was evaluated by Ganda et al. (2016) and it was found suitable in terms of ease of performing and accuracy of results and was in fact comparable with 16S rRNA sequencing results. With more progress in such systems, mastitis diagnosis will become more feasible on farm. Teat microbiota were studied to find their association with mastitis, and significant differences were observed in the bacterial community of teats with different disease status and disease history. Such prophylactic measures can be used to improve health in dairy herds (Falentin et al. 2016), thus improving farm productivity as well as animal welfare.

A magnetic nanoparticle-based calorimetric assay was developed to detect levels of plasmin in milk. It is a heat-stable endogenous serine protease which is released regardless of pathogen involved. This newly developed biosensor has the ability to detect mastitis status with high sensitivity and has the potential to be used as an initial diagnostic test (Chinnappan et al. 2017).

Another biosensor for the rapid detection of *S. aureus* from mastitic milk was designed. It comprised of renewable micro-columns, secondary antibodies for selective binding of target bacteria, and conjugated fluorescence marker (Peedel and Rinken 2014). A magneto-resistive cytometer provided with a microfluidic sample was used for detection of *Staphylococcus* species based on joining of magnetic nanoparticles and specific antibodies. It proved effective when compared with routine PCR analysis; however, further studies are recommended for improving specificity and sensitivity of this rapid biosensor method (Duarte et al. 2017).

## Conclusion

Considerable progress and investment has been made in the last few years in the area of biosensor technology for better diagnosis and maintenance of healthy dairies. Steeneveld et al. (2015) studied the economic consequences and related

expected productivity, but unfortunately most of these have not materialized and are not used at the farm level. In order to enhance the impact of these technologies, their utility should be increased by providing more awareness and guidance to use these novel advanced systems. We understand the detection of mastitic pathogens is extremely difficult due to various factors we discussed earlier; however, it is still highly important and worth doing. The combination of both genomic and proteomic approaches, together with further involvement of nanotheranostic technologies and other sensors, will assist in the quest of better mastitis diagnostic tools.

**Acknowledgments** Authors are thankful to Dr. Peter Thomson, Faculty of Veterinary Science, The University of Sydney, Australia for proof reading of paper.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This study did not require official or institutional ethical approval.

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