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Invited Review Article

Biosensor-based approach to detect bovine mastitis

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Abstract

Mastitis is a major concern for the dairy industry in terms of financial losses, animal welfare, and public health issues. To date, the microscopic evaluation of milk somatic cell counts (SCC) and California mastitis test are routinely employed to detect mastitis at farm level. However, the research has been carried out to develop a highly sensitive diagnostic approach for early and quick identification of udder infection to create an effective therapy program. The biosensor-based approach to detecting mastitis has been a promising area of research during the last decade. Some biosensors for detecting SCC in milk were commercially available. But there are some other areas we need to explore for the development of biosensors, such as enzymes and acute phase proteins that are reported to increase during mastitis. Nanoparticle and aptamer-based biosensors (aptasensors) are also emerging as a laboratory "on a chip" to detect mastitis with high sensitivity and rapid detection rate. Extensive field trials are required for further validation of these biosensors.

Keywords: Acute phase proteins, Biosensor, Enzymes, Mastitis, Milk somatic cells, Nanoparticle

Highlights

- Biosensors are sensitive devices that detect biological molecules in a sample with high specificity and shorter duration through a transducer.
- The biosensors for mastitis are designed to detect somatic cell counts, enzymes, acute phase proteins and antimicrobial peptides in milk.
- Some biosensors for detecting SCC in milk were commercially available.
- Nanoparticle and aptamer-based biosensors (aptasensors) are also emerging as a laboratory "on a chip" to detect mastitis.
- Extensive field trials are required to validate the efficacy of these biosensors.

INTRODUCTION

Mastitis poses major problems for dairy farmers in terms of financial losses, animal welfare, and public health issues (De Vliegher *et al*., 2012). Due to the lack of observable clinical signs, the subclinical form of mastitis is more harmful than the clinical form (Ashraf and Imran, 2018). The economic losses due to mastitis in India accounted for about Rs. 7165.5 crores, of which sub-clinical mastitis contributes Rs. 4151.1 crores (Bansal and Gupta, 2009) associated with a 21% annual decline in milk output (Bardhan, 2013). It is challenging to diagnose the subclinical type of mastitis, and as of now, the majority of diagnoses are made using the traditional procedures of somatic cell count (SCC), California mastitis test, and surf field mastitis test under farm conditions (Ashraf and Imran, 2018). However, these methods require well-trained personnel, and neither of these tests can identify the causative agents (Viguier *et al*., 2009). Thus, efforts are focused on creating a trustworthy and highly sensitive diagnostic approach for early and quick identification of udder infection as well as the causative agents to create an effective therapy program (Mahmmod, 2013). In modern dairy farms, biosensor-based tools are applied for the detection of mastitis based on microbiological and molecular data. Additionally, these biosensor-based instruments can be used with automated milking systems for routine analysis, data extraction, and reporting (Ashraf and Imran, 2018). The present review is aimed to highlight the potential of using biosensors in the diagnosis of bovine mastitis.

Biosensors for mastitis: Basic principle

A biological sensor (biosensor) is a device that can convert a measurable biological response into an electrical signal in the form of data with the help of an integrated transducer that interacts with the biological elements like enzyme, antibody, nucleic acid etc. (Martins *et al*., 2019). The transducing principles are based on the types of targeted bio-molecules and categorized into optical (Yoo and Lee, 2016), electrochemical (Zhang *et al.*, 2019), fibre

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optic (Dudak and Boyaci, 2009), piezoelectric (Pohanka, 2018) and others (magnetic, calorimetric and acoustic) (Umesha and Manukumar, 2018). The transducers should have a recognition element called a probe. In bacterial infection, enzymes, antibodies or oligonucleotides are considered recognition elements (Morales and Halpern, 2018). In mastitis detection, recognition elements for the development of bio-sensors are enzymes such as N-acetyl-b-D-glucosaminidase (NAGase), lactate dehydrogenase (LDH) and catalase (Martins *et al*., 2019), acute phase proteins such as haptoglobin (Hp) and serum amyloid A (SAA) (Pyörälä *et al*., 2011), and antimicrobial peptide such as cathelicidins (Addis *et al*., 2016). The performance of a sensor system is dependent on the quality of the transducer and can be evaluated in an experimental setting by its repeatability and reproducibility (Kamphuis *et al*., 2013). In repeatability, the similarity of successive measurements by a sensor is compared, whereas, in reproducibility, successive results from other systems under different circumstances are compared (Kamphuis *et al*., 2013; Hogeveen *et al*., 2021).

Biosensors to detect somatic cells in milk

The somatic cell counts (SCC) in the milk are the gold standard for the detection of mastitis. SCCs are part of the cellular defence of the mammary gland that includes leukocytes, i.e., polymorphonuclear neutrophils (PMN), macrophages, and lymphocytes, together with a small percentage of epithelial (Paape *et al*., 2002). A normal, i.e., uninfected quarter generally contains below 1 $x10^5$ cells/mL of milk (Hillerton, 1999), which can go up to several million per mL of milk (Mukherjee and Das, 2019).

There are some commercially available somatic cell counters to detect somatic cell counts in milk samples to evaluate udder infections. The basic principle behind these biosensors is to stain the nucleus of the cell with some fluorescent dye and measure the signal.

DeLaval™ cell counter uses propidium iodide (PI) as the fluorescent stain to visualize cell nuclei (Gonzalo *et al*., 2006). It is a battery-operated optical cell counter that can give results within a minute. It was standardized for cow and ovine milk for the detection of SCC in milk (DeLaval, 2005; Gonzalo *et al*., 2006).

Fossomatic™ cell counter uses ethidium bromide to stain the nuclear DNA of somatic cells, and the fluorescence is amplified and recorded. It is used to detect SCC in raw milk of cows and sheep (Schmidt-Madsen, 1979; Gonzalo *et al*., 2003).

Qscout™ is a portable device that provides the SCC along with differential leukocytes such as polymorphonuclear cells (PMN), macrophages and lymphocytes (Leitner *et al*., 2003; Godden *et al*., 2017).

Septer™ is a commercially available SCC counter that evaluates SCC by Coulter counter method (Martins *et al*., 2019).

Enzymes and acute phase proteins concerning mastitis

The pathogenic invasion and their subsequent multiplications in the udder initiate an inflammatory response (Harmon, 1994) and release of certain inflammatory mediators along with changes in capillary permeability (Wellnitz and Bruckmaier, 2012). There is a rapid influx of polymorphonuclear neutrophils in the mammary gland as the first line of defence, which results in increased somatic cell counts (SCC) (Wickström *et al*., 2009) and to date, the SCC is the primary indicator for udder health (Dufour and Dohoo, 2013). The tissue damage during mastitis also induces an acute phase response that leads to the production of acute phase proteins (APPs) from the hepatocytes (Ali *et al*., 2023). These acute phase proteins can cross the blood-milk barrier due to altered permeability of the membrane and come into milk (Wellnitz and Bruckmaier, 2012). The damaged tissue also secretes enzymes like NAGase and LDH, which also have a significant correlation with udder health status (Zank and Schlatterer, 1998). The relevant APPs for mastitis are haptoglobin (Hp), serum amyloid A and alpha1 acid glycoprotein, which can be detected in both serum and milk (Eckersall *et al*., 2001). The sensitivity and specificity of haptoglobin biomarkers are 82% and 94%, respectively in serum and 86% and 100% in milk (Eckersall *et al*., 2001). The sensitivity and specificity of serum amyloid A were reported to be 83% and 90% in serum and 93% and 100% in milk (Eckersall *et al*., 2001).

Enzyme-detecting biosensors for mastitis

β-N-acetylglucosaminidase (NAGase): NAGase is considered a potential mastitis biomarker released in milk from the damaged epithelial cells of the mammary gland (Wellnitz and Bruckmaier, 2012). An electrochemical assay to detect NAGase using screen-printed carbon electrodes was developed in the laboratory (Pemberton *et al*., 2001). In this assay, enzyme substrate 1-naphthyl-N-acetyl-β-D-glucosaminid was added to the biological samples with NAGase and hydrolysed to 1-naphthol that was monitored amperometrically. The assay had a short reaction time, and the results could be obtained within 100 seconds. Though the assay was standardized under laboratory conditions and never tested in field conditions, it can be considered the first approach to develop a sensor device for electrochemical NAGase sensors.

Another Biacore system was developed to detect NAGase in milk based on the optical phenomenon of surface plasmon resonance (SPR) resulting from the

interaction of proteins with other biomolecules like proteins, peptides, nucleic acid, lipids etc. (Welbeck *et al*., 2011). In this Biacore system, one of the ligands is immobilized on a sensor chip, and the analyte is allowed to pass the surface through a microfluidic system. The interaction of ligands with the analyte changes the angle of light reflection, and the degree of this change is proportional to the amount of analyte mass on the chip surface (Murphy *et al*., 2006). The advantages of the Biocore system are low reagent requirement, unlabeled antibodies and it is reusable. The detection limit was 10 U/mL. The assay also had a linear response of 2.5-400 U/L compared to 3.1-108 U/L in screen-printed carbon electrode assay (Murphy *et al*., 2006). However, the assay is yet to be tested in real mastitis samples.

Lactate dehydrogenase (LDH): A screen-printed amperometric LDH biosensor was developed based on the electrocatalytic reaction of LDH to generate NADH and its subsequent oxidation by 3,4-dihydroxybenzaldehyde (3,4-DHB) electropolymerized on the carboneous working electrode (Young Hong *et al.*, 2002). The detection limit of this sensor was reported to be 50 U/L.

Another quantum dots (QDs) based biosensor has been developed to detect lactate dehydrogenase (LDH) by evaluating the fluorescence of QDs (Ren *et al*., 2010; Yang *et al*., 2011). The principle of this system is the quenching of fluorescence intensities in the QDs by nicotinamide adenine dinucleotide (NAD, the coenzyme of LDH), followed by its intensification by LDH that consumes NAD. The detection limit of this biosensor is 75 U/L, which is within the reported LDH level in mastitis (Hiss *et al*., 2007). This biosensor was successfully tested with human serum samples, and the data obtained was comparable with the data obtained from the bioanalyzer (Yang *et al*., 2011).

Catalase: Catalase is an antioxidant enzyme present in a variety of cells. Being an oxidoreductase, it degrades hydrogen peroxide into water and oxygen. Catalase is released in milk by somatic cells, and its level is correlated with milk SCC (Hamed *et al*., 2008). Therefore, it can also be considered a biomarker for mastitis (Andrei *et al*., 2016).

An amperometric biosensor for the detection of catalase in bovine milk has been developed after immobilizing the catalase on a thin layer surface (Futo *et al*., 2012). The enzyme is then allowed to degrade hydrogen peroxide to detect the infection status of milk. The linear measuring range of this sensor was reported to be 0.1 mmol/L.

Acute phase protein detecting biosensors

Haptoglobin (Hp): Hp is a specific biomarker of mastitis. The Hp level in the milk of healthy quarter is extremely low or even undetected (Eckersall *et al*., 2001), but its level is increased by several folds during udder infection or mastitis (Gro¨nlund *et al*., 2003). Therefore, Hp is a target biomolecule for mastitis detection through biosensors.

The first ever reported biosensor assay to measure milk was developed by Akerstedt *et al*. (2006). In this assay, Hp is immobilized to a CM5 sensor chip, and milk samples are mixed with bovine haemoglobin before being injected into the sensor surface. The presence of Hp in milk samples forms a complex with added haemoglobin and prevents the binding of haemoglobin to the Hp coated on sensor chips. The detection principle is based on the changes in the refractive index at the sensor surface. The detection limit of this Hp biosensor assay is 1.1 mg/L. This assay was compared with the conventional ELISA-based commercial kits for mastitic milk samples, and the results were comparable.

An amperometric immunosensor for the detection of Hp in milk was developed after immobilizing anti-Hp antibodies on the gold electrode (AuE) surface (Tan *et al*., 2012). In this assay, Hp in the milk samples forms a complex with anti-Hp antibodies that blocks the electron transfer on the gold electrode (AuE) surface and decreases the amperometric response. The level of Hp in the milk is detected by observing the amperometric current. The assay had a linear response of Hp within the range of 15-100 mg/L, and the detection limit was 0.63 mg/L. There were no significant differences between the results obtained from the biosensor and other commercial kits for mastitic milk samples.

Serum amyloid A (SAA): Serum amyloid A (SAA) is also a potential biomarker for mastitis detection. The sensitivity of SAA detection in serum and milk for mastitis is 83% and 93%, respectively (Eckersall and Bell, 2010). The estimation of SAA also has an added advantage in that its concentration is altered according to the type of infection. The concentration of SAA is highest in *E. coli* infections compared to Streptococci and Staphylococci (Trela *et al*., 2022). Therefore, SAA can also be targeted for biosensor development.

Balayan *et al*. (2022) developed another molecular imprinting-based biosensor for SAA and validated it in neonatal sepsis patients (Balayan *et al*., 2022). In this sensor, multi-walled carbon nanotubes (MWCNTs), manganese oxide nanospheres (MnO_2NSs), and cobalt oxide nanoparticles (Co_3O_4NPs) were coated on the screen-printed electrode (SPE). Molecularly imprinted

polymer (MIP) specific to SAA was generated with a template, monomer, and crosslinker in porogen solvent and specific cavities were created by the polymerization of the monomer around a template. The complementary binding sites were obtained when the specific templates were removed and cats as the binding sites for SAA. This biosensor had a detection limit of 0.01 pM to 1 μM with a response time of less than 3 minutes. However, this biosensor has yet to be tested in mastitis samples (Trela *et al*., 2022).

Nanotechnology-based biosensors

The idea of laboratory "on a chip" emerged after the utilization of nanomaterials to detect infectious agents in biological fluids. The plasmonic shift of nanoparticles and changes in magnetic properties enable the detection of causative pathogens, antigens and toxins with good sensitivity (Driskell and Tripp, 2009). A nanoparticle-based calorimetric method to detect bovine mastitis has been developed (Chinnappan *et al*., 2017). In this method, plasmin was detected in bovine milk as it was reported that plasmin increases during mastitis and causes proteolysis of milk proteins such as casein. In this sensor, the substrate of plasmin was coupled to magnetic nanoparticles in the form of a black-coloured monolayer on a gold sensor surface. The presence of plasmin in the milk cleaves the substrate, and the peptide fragments are then attached with magnetic beads. The beads are then attracted by the magnets present under the gold sensor and the sensor surface is turned from black to golden colour. The area of the golden colour surface is proportional to plasmin activity. The sensitivity of this method was 1 ng/mL of plasmin *in vitro*. This biosensor was tested with mastitis-positive samples, and the infection was confirmed. However, extensive field trial is required to validate its efficacy.

Another colloidal gold nanoparticle-based immunochromatographic strip (ICS) for the detection of *S. aureus* in mastitic milk has been developed by Nagasawa *et al*. (2020). In this technique, ribosomal protein-L7/L12 was detected in milk by anti-RP-L7/L12-coated colloidal gold nanoparticle-based immuno-chromatographic tests. The ICS reacted with *S. aureus*, and there was a positive correlation between the *S. aureus* (*nuc* gene) copy and ICS test scores. The advantage of this sensor is that it can detect *S. aureus* infection. Nanoparticle-based biosensor for detecting catalase in milk was developed with a nanocomposite of polyaniline (PAni)/gold nanoparticles (AuNP) with a lower detection limit of 2.34 μM and a recovery rate of 99.6% (Domínguez-Aragón *et al*., 2021).

Aptamer based biosensor

Aptamers are ssDNA that can bind with proteins and other small molecules. They are used like antibodies in surface plasmon resonance (SPR) based biosensors. These biosensors are called aptasensors. The aptamer-based biosensors are small in size, chemically stable, cost-effective, and have good specificity (Song *et al.*, 2008). Thus, these biosensors have been used for environmental monitoring (McConnell *et al*., 2020), detection of mycotoxins (Guo *et al*., 2020) and therapeutics (Ning *et al*., 2020). One such SPR-based biosensor has been developed for the detection of catalase in bovine milk after immobilizing a biotin-tagged aptamer onto a gold surface with a linear range between 20.5 and 1000 nM and good specificity for catalase (Ashley and Li, 2013).

Conclusions

A variety of diagnostic tools are available to detect mastitis, but bio-sensing tools for early detection of mastitis are a promising area of research. These biosensors can be employed either for on-farm detection of mastitis or can be implemented with an automatic milking system for routine surveillance. Bio-sensing devices to detect mastitis markers, such as enzymes, acute phase proteins, and antimicrobial peptides in milk, have been developed, but extensive field trials are required to validate the results with high accuracy. Nanoparticle and aptamer-based biosensors (aptasensors) are the latest trend in this field, which could emerge as laboratory "on a chip".

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Author's contributions: JM, KD: Conceived the idea; DB, PRG and PKD: Collected the information; JM: Drafted the manuscript; KD, PKD, DB: Corrected the manuscript.

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