Review

Mastitis detection: current trends and future perspectives

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Bovine mastitis, the most significant disease of dairy herds, has huge effects on farm economics due to reduction in milk production and treatment costs. Traditionally, methods of detection have included estimation of somatic cell counts, an indication of inflammation, measurement of biomarkers associated with the onset of the disease (e.g. the enzymes N-acetyl-β-D-glucosaminidase and lactate dehydrogenase) and 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. Recently, significant advances in the identification of nucleic acid markers and other novel biomarkers and the development of sensor-based platforms have taken place. These novel strategies have shown promise, and their advantages over the conventional tests are discussed.

Introduction

Bovine mastitis (*mast* = breast; *itis* = inflammation), a major disease affecting dairy cattle worldwide, results from the inflammation of the mammary gland. The severity of the inflammation can be classified into sub-clinical, clinical and chronic forms, and its degree is dependent on the nature of the causative pathogen and on the age, breed, immunological health and lactation state of the animal. Sub-clinical mastitis is difficult to detect due to the absence of any visible indications, and it has major cost implications. Chronic mastitis is a rarer form of the disease but results in persistent inflammation of the mammary gland. Currently, milk quality payments are based on somatic cell counts (SCCs), and elevated levels result in reduced payments. This, in addition to reduction in milk volume and treatment costs, significantly affects farm incomes [1] (see also Box 1). This review gives a brief overview of the development of the inflammation and critically assesses recent advances in mastitis diagnostics with particular focus on their potential in diagnosing mastitis at an early stage and 'on-site'.

Pathogenesis

A comprehensive understanding of the pathogenicity of mastitis is key for the development of appropriate detection techniques. The primary cause of mastitis is a wide spectrum of bacterial strains; however, incidences of viral, algal and fungal-related mastitis were also reported [2].

Normally, the teat canal is tightly closed by sphincter muscles, preventing the entry of pathogens. It is lined with keratin, a waxy material derived from stratified squamous epithelium that obstructs the migration of bacteria and contains antimicrobial agents, such as long-chain fatty acids, that assist in combating the infection. However, the efficiency of keratin is restricted [3–5]. Fluid accumulates within the mammary gland as parturition approaches, resulting in increased intramammary pressure [5] and mammary gland vulnerability caused by the dilation of the teat canal and leakage of mammary secretions [6]. Additionally, during milking, the keratin is flushed out and there is distention of the teat canal [7]. The sphincter requires ~2 h to return back to the contracted position [4].

Once inside the teat, bacteria must also elude the cellular and humoral defence mechanisms of the udder [6]. If they are not eliminated, they start multiplying in the mammary gland (Figure 1). They liberate toxins and induce leukocytes and epithelial cells to release chemoattractants, including cytokines such as tumour necrosis factor-α (TNFα), interleukin (IL)-8, IL-1, eicosanoids (like prostaglandin F2α [PGF2α]), oxygen radicals and acute phase proteins (APPs) (e.g. haptoglobin [Hp], serum amyloid A [SAA]). This attracts circulating immune effector cells, mainly polymorphonuclear neutrophils (PMNs), to the site of infection [8–11].

PMNs act by engulfing and destroying the invading bacteria via oxygen-dependent and oxygen-independent systems. They contain intracellular granules that store bactericidal peptides, proteins, enzymes (such as myeloperoxidase) and neutral and acidic proteases (such as elastase, cathepsin G, cathepsin B and cathepsin D) [12,13]. The released oxidants and proteases destroy the bacteria and some of the epithelial cells, resulting in decreased milk production and release of enzymes, such as N-acetyl-β-D-glucosaminidase (NAGase) and lactate dehydrogenase (LDH) (Figure 1). Destruction of most of the PMNs takes place by apoptosis once their task is fulfilled. Subsequently, macrophages engulf and ingest the remaining PMNs [9,14]. The dead and sloughed off mammary epithelial cells, in addition to the dead leukocytes, are secreted into the milk, resulting in high milk SCCs.

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Box 1. Impact of mastitis

Direct effects [1,76]

- Temporary or permanent loss in milk production.
- Poor milk quality, for example reduction in milk fat content, resulting in dairy products with less favourable organoleptic properties.
- Reduction in price due to high somatic cell count.
- Loss due to discarding of milk after the antibiotic treatment.
- Additional treatment costs related to, for example, drugs and veterinary care.
- Increased labour costs, for example extra labour required for husbandry of cattle and for application of preventive measures.
- Increased costs for surveillance of milk quality and disease status among rest of the herd.
- Premature culling or reduced productive-life of cattle.
- Lower value for culled cattle meat because the carcass yield and quality is reduced.

Financial costs [76–78]

- In the US, the projected annual losses caused by mastitis are US$2 billion.
- In the UK, mastitis causes an annual loss of approximately £300 million to dairy farmers.
- In Northern Ireland, the cost of clinical mastitis for an average 100-cow herd is £5000 per year, with total mastitis infections costing £14 million annually.
- In the Republic of Ireland, the cost of clinical mastitis is approximately €693 per year for every infected cow.
- In the Netherlands, the average cost per infected cow varies between €164 and €235.

If the infection persists, internal swelling within the mammary epithelium, not normally detectable by an external examination, can occur. The mammary gland alveoli become damaged and start losing anatomical integrity (Figure 1). The blood–milk barrier is breached, causing extracellular fluid components, such as chloride, sodium, hydrogen, potassium and hydroxide ions, to enter the gland and mix with the milk [10].

When extensive damage to the blood–milk barrier has occurred, blood might be detected in the milk. This leads to visible changes on the udder, such as enhanced external swelling and reddening of the gland. Changes also occur in the milk, including increased conductivity, increased pH, raised water content and the presence of visible clots and flakes [10,15–17]. This marks the initial stage of clinical symptoms, and the most severe infections might ultimately result in the death of the animal.

Current approaches for diagnosis of mastitis

Early diagnosis is of the utmost importance due to the high costs of mastitis. European Union legislation (Regulation 853/2004) stresses that milk selected for human consumption must originate from healthy animals. Diagnostic methods have been developed to check the quality of the milk through detection of mammary gland inflammation and diagnosis of the infection and its causative pathogens. Currently, assays often used include measurement of SCCs, enzymatic analysis and the California milk clotting

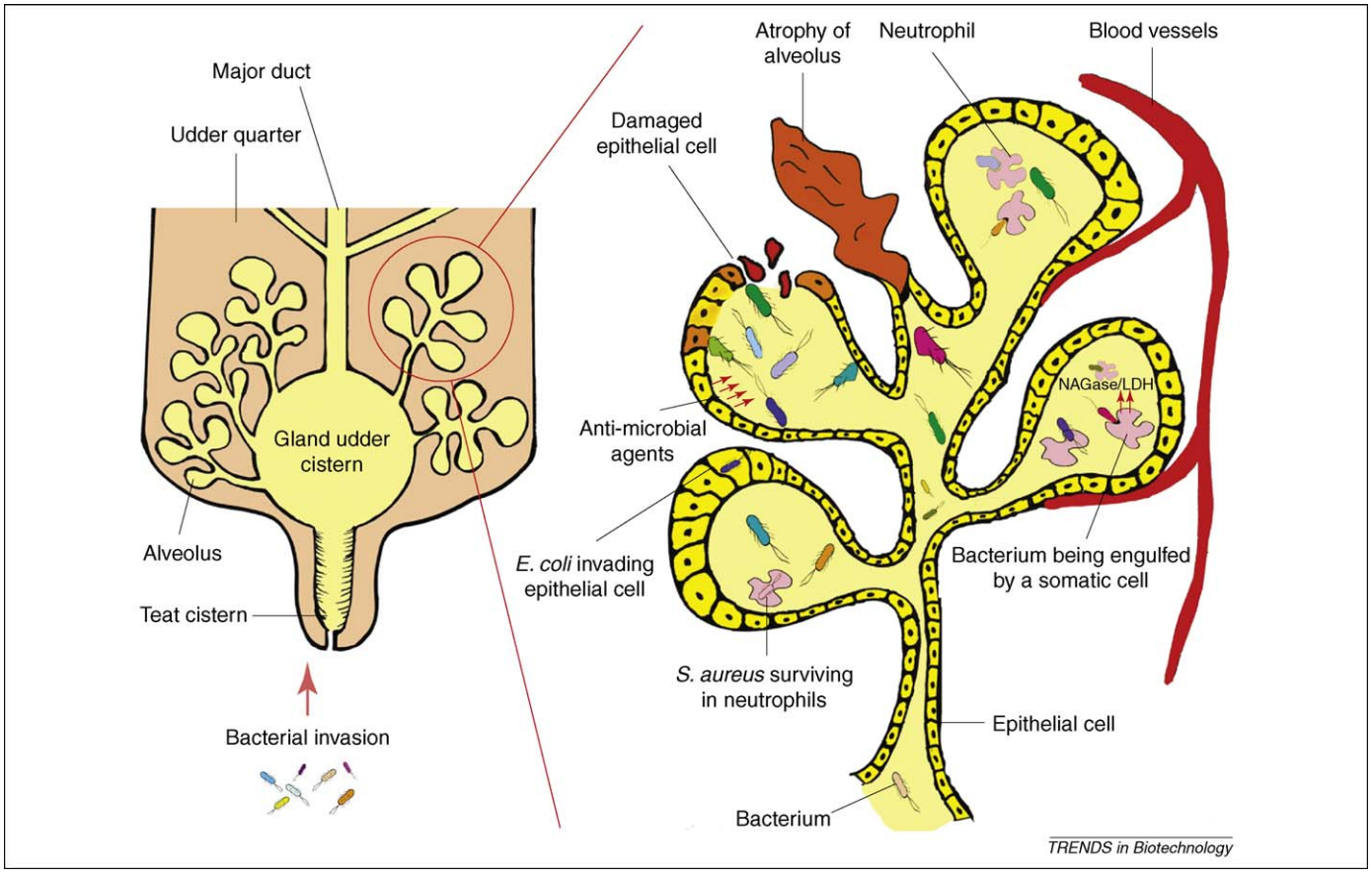

Figure 1. Schematic representation of mastitis development in an infected udder. Environmental and contagious microorganisms invade the udder through the teat cistern. They then multiply in the udder where they are attacked by neutrophils while damaging the epithelial cells lining the alveoli, with subsequent release of enzymes like NAGase and LDH. The epithelial cells also secrete anti-microbial compounds. Considerable tissue damage is observed once the immune effector cells begin to combat the invading pathogens.

Box 2. Current SCC and alternative methods for detection of mastitis

California mastitis test (CMT)
This assay indirectly measures the SCC in milk samples. A bromocresol-purple-containing detergent is used to break down the cell membrane of somatic cells, and the subsequent release and aggregation of nucleic acid forms a gel-like matrix with a viscosity that is proportional to the leukocyte number.
- Advantages: cost effective (~US$12 for 350 tests), rapid, user friendly and can be used 'on-site' or in the laboratory.
- Disadvantages: can be difficult to interpret and has low sensitivity.

Portacheck
This assay uses an esterase-catalysed enzymatic reaction to determine the SCC in milk.
- Advantages: cost effective (~US$3 per test), rapid and user friendly.
- Disadvantage: low sensitivity at low SCCs.

Fossomatic SCC
This counter operates on the principle of optical fluorescence. Ethidium bromide penetrates and intercalates with nuclear DNA, and the fluoresecent signal generated is used to estimate the SCC in milk.
- Advantages: rapid and automated.
- Disadvantages: the device is expensive (~US$7000) and complex to use.

Delaval cell counter
This counter operates on the principle of optical fluorescence, whereby propidium iodide is used to stain nuclear DNA to estimate the SCC in milk.
- Advantages: rapid and the device is easily transportable.
- Disadvantage: relatively expensive.

Electrical conductivity (EC) test
This test measures the increase in conductance in milk caused by the elevation in levels of ions such as sodium, potassium, calcium, magnesium and chloride during inflammation.
- Advantage: can be used 'on-site'.
- Disadvantage: non-mastitis-related variations in EC can present problems in diagnosis.

Culture tests
Laboratory-based tests use selective culture to identify different microorganisms involved in causing mastitis.
- Advantage: identifies specific pathogens causing mastitis.
- Disadvantages: cannot be used 'on-site' and the waiting time for results can be days.

pH test
The rise in milk pH, due to mastitis, is detected using bromothymol blue.
- Advantages: user friendly, cost effective and rapid.
- Disadvantage: not as sensitive as other tests.

Enzymes
Assays are used to detect enzymes, such as NAGase and LDH.
- Advantage: assays are rapid.
- Disadvantage: assays might be laboratory-based.

test [2]. In Europe, elevated SCCs above 200 000 cells/mL are widely used as an indicator of mastitis [18] and are determined using haemocytometers or cell counters. Colourimetric and fluorometric assays have been developed for measuring the concentrations of enzymes elevated in milk during mastitis (e.g. NAGase or LDH). Use of culturing techniques for the detection of mastitis-causing microorganisms is still the gold standard, although it is very labour-intensive and therefore expensive.

Mastitis can also be detected using 'cow-side' or 'on-site' tests, which can be used by both farmers and veterinarians and which require relatively little training. One of the oldest and best known is the California mastitis test (CMT) [19]. It is based on the principle that the addition of a detergent to a milk sample with a high cell count will lyse the cells, release nucleic acids and other constituents and lead to the formation of a 'gel-like' matrix consistency. However, the interpretation can be subjective, and this might result in false positives and negatives. Mastitis can also be detected via changes in conductivity or pH (see Box 2). Although these effects are easy to monitor, they are relatively insensitive. Thus, there is a major need for new biomarkers that are specific for mastitis, easy to detect, occur at a very early stage and that can be measured 'on-site'.

Development of new biomarkers for mastitis

Earlier detection of mastitis, and the identification of the associated causative agents, will improve the well-being of animals by allowing timely and efficient treatment. Advances in relevant proteomics techniques, such as two-dimensional gel electrophoresis (2D-GE) and mass spectroscopy (MS) [20–22], have led to the identification of several new proteins involved in mastitis.

Smolenski and colleagues [22] compared a mastitic milk sample to a non-mastitic milk sample using direct liquid chromatography-tandem MS and 2D-GE followed by matrix-assisted laser desorption ionization time-of-flight MS analysis of individual protein spots. Six chaperonins with a role in pathogen recognition were identified only in mastitic samples and therefore have potential as new markers for mastitis. This study also reported the presence of some neutrophil-associated proteins, cathelicidin, peptidoglycan recognition protein, lymphocyte cytosolic protein 1 and the macrophage scavenger receptor, types I and II, for the first time in milk samples [22]. The whey-protein patterns of mastitic milk were also studied. Baeker and coworkers [23] reported the potential use of prostaglandin D synthase as a new marker that is upregulated in mastitic milk but as yet there have been no significant advances in its use. Hogarth and coworkers [24] also demonstrated that the whey from cows affected with mastitis inflammation had increased levels of proteins of blood origin, such as serotransferrin and bovine serum albumin, and reduced concentrations of many of the major milk proteins [24].

Proteomic analysis of bovine neutrophils has resulted in the identification of over 250 proteins, of which 19 are known to be involved in the immune response of the host. They could potentially be used as markers for its detection, as reduced neutrophil function has been correlated with mastitis [20].

A recent study investigated the protein patterns of mammary tissues from healthy and mastitis-infected animals to identify new markers. The results showed that there is an upregulation of κ-casein and a downregulation of cytochrome C oxidase and annexin V in animal tissues that are mastitis-infected [25].

The development of proteome profiles of mastitis-causing pathogens, combined with available information on enzymes, toxins and metabolites produced in the udder, could assist in their identification in milk. To that end, Taverna and coworkers [26] carried out proteomic characterization of a *Staphylococcus aureus* strain isolated from a mastitis case and presented a 2D-GE reference map of surface proteins known to contribute to bacterial adhesion to mammary tissues and to increase bacterial resistance to phagocytosis.

The proteomics studies mentioned above resulted in information on the different protein expression pattern obtained from mastitis-infected milk and on the proteins expressed by invading pathogens. This information can be applied not only to the discovery of new therapeutic targets but also to the search for new diagnostic biomarkers. However, the successful application of these new biomarkers in a detection device still remains a challenge.

Recent laboratory developments in the detection of mastitis

Technological advances, together with increased proteomic and genomic information, have resulted in improvements in the sensitivity of assays used for the detection of mastitis. Immunoassays, such as enzyme-linked immunosorbent assay (ELISA), can provide a reliable and inexpensive approach provided that suitable antibodies are available against specific inflammation-related biomarkers or the causative microorganisms. There have also been significant developments in nucleic-acid-based testing for the identification of the latter.

Immunoassays

More than one hundred known organisms can be responsible for causing mastitis [27], but ELISAs have only been developed for some of the most prevalent pathogens, such as *S. aureus*, *Escherichia coli* and *Listeria monocytogenes*. For example, an *S. aureus* antibody test kit (SAATK) (Veterinary Medical Research and Development [VMRD], Inc., Pullman, WA, USA) was assessed as a primary screen for cows suspected of having an *S. aureus* infection. However, microbial culture of the milk of ELISA-positive cows was required for confirmation [28]. An ELISA to determine the level of antibodies produced against *L. monocytogenes* was also developed [29].

A magnetic-bead-based ELISA was developed for the detection of staphylococci using beads coated with an anti-*S. aureus* monoclonal antibody [30]. This approach has certain advantages over conventional ELISAs, having shorter incubation times, fewer manipulations and requiring smaller volumes of reagents. Flow cytometry was also used to detect antibodies to *S. aureus* in milk. The method gave an earlier result in 25% of cases when compared with bacteriological tests [31].

Numerous immunoassays have been developed for the detection of pathogens in milk [32–35] and are used for monitoring milk quality. However, very few studies have been undertaken for the development of immunoassays that detect pathogens in milk as definitive causative agents of mastitis. Such assays might also be useful for mastitis detection. However, studies to validate this assumption are required.

Immunoassays can also be used to detect inflammation-related biomarkers present in the milk at different stages of sub-clinical mastitis. For example, Hp concentrations have been reported to increase significantly in plasma, as well as in milk, during mastitis and thus Hp was suggested as a potential marker for diagnosis [36,37]. Hiss and coworkers [37] developed an ELISA for its detection, with a detection limit of 0.07 μg/mL in both milk and serum. SAA is another example of an APP marker that shows elevated levels in mastitic milk [38–40]. Szczubial and colleagues [41] were able to detect elevated concentrations of SAA of up to 322.26 μg/mL in mastitic milk (compared with normal levels of 11.67 μg/mL) using a commercially available solid-phase-sandwich ELISA (Tridelta Phase™ range SAA kit, Tridelta Development Ltd, Co. Wicklow, Ireland). This kit consists of an anti-SAA monoclonal antibody that captures SAA from either test samples or standards. This binding event is detected by the addition of streptavidin–horse radish peroxidase conjugate and, subsequently, a tetramethyl benzidine (TMB) substrate, leading to a colour change that is dependent on the concentration of SAA present.

The application of biomarker-based assays, developed within the last decade, has already shown considerable promise for mastitis detection. Nevertheless, additional studies on the validation of these assays for mastitis detection are required.

Nucleic acid testing

The genome sequences of many of the major mastitis-causing pathogens are now available and can be utilized to develop nucleic acid-based testing methods, such as PCR. Such tests are generally more expensive than, for example, immunoassays. However, they are highly sensitive and specific, can be performed rapidly (e.g. 'real-time' PCR) and can overcome the sensitivity and time-constraints sometimes encountered with culture-based tests [42] and thus could complement or replace them in the long-term.

PCRs allow the identification of closely related organisms within a few hours. Multiplex PCR and 'real-time' PCR assays that can simultaneously detect different mastitis-causing organisms in milk samples have been described [43–48], and the most recently developed assay is capable of detecting 11 of the major mastitis-associated pathogens, including *E. coli, S. aureus, Streptococcus agalactiae* and *Streptococcus uberis* [49]. Nucleic acid sequence based amplification (NASBA), which is used for quantification of RNA, has an advantage over PCR methods in that it is capable of discriminating between dead and living organisms, and real-time NASBA for the detection of *Bacillus cereus* in milk has been reported [50]. The application of real-time PCR or NASBA could revolutionize veterinary diagnostics by reducing sample analysis times significantly and allowing the simultaneous analysis of a large number of samples for multiple organisms.

Recent developments in 'cow-side' tests for the detection of mastitis

Rapid, 'cow-side' mastitis tests could be used by farmers and veterinarians to diagnose and treat the inflammation in its early stages, thus having the potential to stop the propagation of the disease in the herd.

An increase in temperature is one of the symptoms associated with mastitis. A thermal camera was used to diagnose experimentally-induced mastitis and could detect temperature changes of 1 to 1.5 °C [51]. Infrared thermography was also used to measure skin surface temperatures in infected cows, and a strong correlation ($R^2 = 0.92$) between skin surface temperature and SCCs was observed [52]. This non-invasive approach can be used 'on-site'. However, the ambient temperature can affect this assay, and a rise in temperature might only occur in some cases of mastitis; therefore, temperature might only act as an indicator of infection.

Estimation of the levels of inflammation-related enzymes might also be used for the detection of mastitis as these show good correlation with SCCs. For example, an LDH activity assay was carried out by Hiss and coworkers [53] using dry chemistry and a portable spectrophotometer with comparable variation coefficients to the assays performed in a laboratory environment. Other enzymatic tests include the detection of an esterase secreted by somatic cells using an enzymatic assay on a dipstick[*]. Bioluminescence-determination assays, based on estimation of the ATP concentrations in somatic cells [54] or the recognition of somatic cell DNA by fluorescent staining[†], can also be used 'on-site' for the reliable determination of elevated SCC levels and thus the probable presence of mastitis.

Current and new trends in mastitis detection during automatic milking

Robotic milking has increased significantly over the past 15 years, and 4% of Dutch farmers now use this method [55]. It provides an ideal format for 'on-line' mastitis monitoring and, therefore, reliable and sensitive methods are necessary (Figure 2).

Any 'on-line' mastitis detection is currently performed using electrical conductivity (EC), SCCs or colour determination, with milk EC being the most commonly used 'on-line' test. However, although a change in conductivity might be a useful indicator, on its own it is not a reliable or sensitive parameter for conclusive diagnosis [56,57]. Milk colour analysis has also been used in automatic milking systems for the indication of mastitic infection [58], as the presence of a yellow colour or of blood in the milk, might be highly indicative of mastitis. However, the milk fat content can also influence colour, and some colour sensors failed to detect sub-clinical mastitis [56]. Therefore, the development of novel sensors with higher sensitivities is the goal of many recent research efforts.

For example, Mottram and coworkers [59] evaluated a chemical-array-based sensor, termed an 'electronic tongue', that was able to detect chloride, potassium and sodium ions released during mastitis in addition to inorganic and organic cations and anions (Figure 2). This sensor could successfully discriminate between normal and mastitic milk samples with a specificity and sensitivity of 96% and 93%, respectively. Eriksson and coworkers [60] demonstrated that milk from mastitic and healthy cows could be distinguished using a gas-sensor-array system, or 'electronic nose'. It consisted of several gas sensors that interact with volatile substances, including sulphides, ketones, amines and acids (Figure 2). More recently, Hettinga and colleagues [61] were able to identify different pathogens, such as *S. aureus*, coagulase-negative staphylococci, streptococci and *E. coli*, and to determine infection-free udder quarters based on the detection of the patterns of volatile metabolites produced (Figure 2). Elevated levels of lactate can also be used for the detection of early stages of mastitis. Limitation of oxygen availability in the mammary glands will lead to increases in the levels of lactate that are directly proportional to the level of metabolic activity. Lactate concentrations detected during mastitis infection showed positive correlation with SCCs [62]. A lactate screen-printed sensor that contains lactate oxidase printed onto the sensor surface has already been developed [63]. Here, lactate oxidase reduces lactate and produces electrons, which generates a current that is measured using a potentiostat and that can be correlated to the concentration of lactate present. These sensors have particularly promising potential as 'on-line' sensors because they are more sensitive, but their ability to detect sub-clinical mastitis is yet to be demonstrated.

Biosensors have also been developed to detect mastitis. They use a biological receptor molecule (e.g. antibody, enzyme, nucleic acid) in combination with a transducer to produce an associated signal, allowing observation of a specific biological event (e.g. an antibody–antigen interaction). For example, Pemberton and coworkers developed an electrobiochemical sensor using a screen-printed carbon electrode (SPCE) that could detect NAGase [64] via its ability to convert the substrate 1-naphthyl N-acetyl-β-D-glucosaminidine to 1-naphthol, which was subsequently detected by the electrode [65]. The limit of detection of this NAGase assay is 10 mU/mL.

In another approach, Akerstedt and colleagues [66] developed a competitive biosensor assay using surface plasmon resonance to monitor the interaction between Hp, which was immobilized onto the chip surface, and haemoglobin (Hb) to discriminate between sub-clinical mastitic and non-mastitic milk (Figure 2). Hp binds strongly to Hb. Therefore, by mixing the milk sample with Hb, any Hp present in the milk sample will bind to the Hb, thus preventing the binding of the Hb to the immobilized Hp. In the absence of Hp (i.e. in an uninfected milk sample), Hb will bind to the immobilized Hp, resulting in a positive signal, which is reduced in correlation to the levels of Hp present in the milk. Milk samples that contain any blood cannot be analysed using this method. However, blood in milk does not normally occur in the sub-clinical stage of mastitis, and this method could still be used in its detection.

[*] Leslie, K. *et al.* An Evaluation of the PortaSCC® Test as a Measure of Udder Health Status Dairy Cows (an excerpt from a technical report), http://www.portacheck.com/guelph.php.

[†] See, for example, the DeLaval Cell Counter DCC, http://www.delaval-us.com/Products/Milking/Cell-counter-DCC/DCC/default.htm.

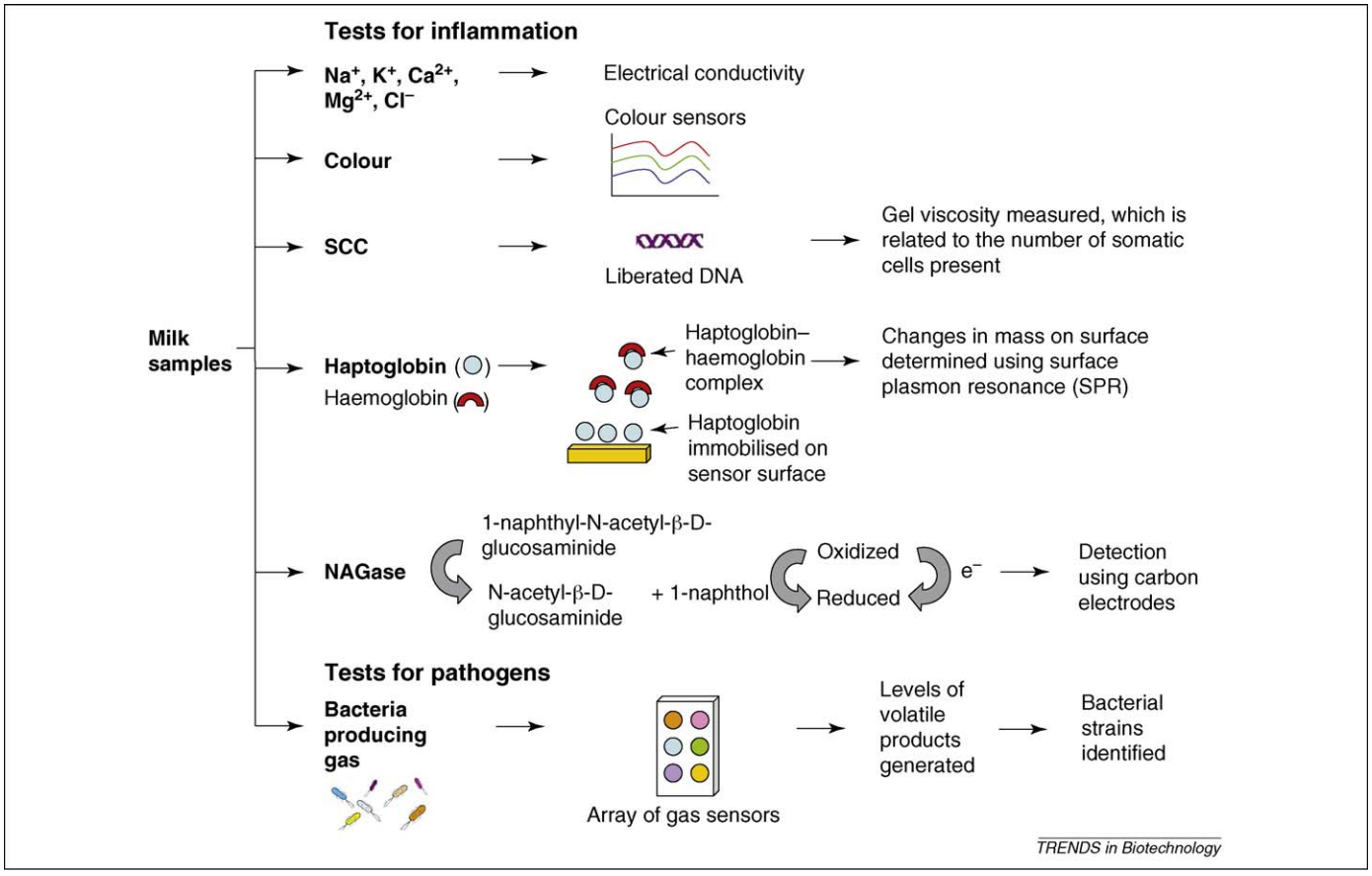

Figure 2. Current and potential 'on-line' assays for the detection of mastitis in milk. The colour, electroconductivity and the SCC sensors are currently used as 'on-line' assays, whereas the sensors for the detection of NAGase, haptoglobin and gases produced by bacteria have yet to be incorporated 'on-line'. These sensors show great potential for the accurate detection of mastitis.

Whyte and coworkers [67] developed a method to automatically determine the SCC based on measuring the DNA content of somatic cells (Figure 2). After somal cell lysis, the liberated DNA and histones form a gel-like complex with a viscosity proportional to the amount of DNA and histones released; the DNA and histone levels can then be measured and correlated to the SCC. In an alternative method, the DNA from somatic cells was incubated with PicoGreen, and the resulting fluorescence was measured using an optical sensor. This assay showed good correlation to Fossomatic determination of SCC ($R^2 = 0.918$). However, the cell-lysis step must be automated before this sensor can be used 'on-line' [68].

These sensors described above have the potential to clearly discriminate between sub-clinical and non-mastitic milk. The combination of such sensor-based platforms with the development of novel biomarkers could thus allow the diagnosis of the pre-clinical stage of mastitis before significant loss in milk production occurs.

Advances in microfluidics and their potential for mastitis detection

Recent advances in microfluidics and so-called 'biochips' (which are also referred to as a 'lab[oratory]-on-a-chip') have the capacity to revolutionize diagnostics [69], and these technologies have already been applied for the detection of mastitis.

Moon and colleagues [70] developed disposable microchips to be used with a portable reader system to measure milk SCC. The milk sample is mixed with a lysis solution to burst the somatic cells, and a fluorescent dye is added to stain the DNA. The sample is then applied to the microchip, which uses a capillary flow to allow even distribution of the sample, and the fluorescence is measured with the portable reader system. This assay showed good correlation with commercial tests for SCCs. Similarly, another disposable device that can detect mastitis based on counting milk leukocytes was recently reported [71]. Here, the milk is carefully mixed with a meta-chromatic substance to stain the leukocytes. The somatic cells are distributed evenly in the chip by capillary action, and the stained cells are then visually identified using fluorescence microscopy. This device has the advantage of having different reaction chambers, allowing the milk to be mixed with the dye on the chip, thus making the device even more user-friendly. Garcia-Cordero and colleagues [72] developed a rapid, low-cost microfluidic CD-based assay device for determining SCCs in which milk samples are applied to a plastic disk with funnel-shaped channels. After centrifugation on a conventional CD-player, the SCC can be measured based on the height of the cell pellet formed. This approach showed excellent correlation with SCC levels determined using conventional approaches.

Choi and coworkers [73] designed a chip for simultaneously monitoring pathogens, somatic cells and pH in raw milk samples. Antibodies against pathogens and somatic cells were immobilized on the chip and antigen–

antibody complexes formed are detected using fluorescence microscopy. The pH is measured by monitoring the fluorescence change of a hydrogel-entrapped pH indicator. Chip technologies could also be applied to the detection of causative pathogens. For instance, Lee and coworkers [74] developed a biochip that incorporated DNA amplification of genes that are specific for seven known mastitis-causing pathogens. A similar microfluidic device that integrates solid-phase extraction and NASBA has recently been reported for the identification of low numbers of *E. coli* [75].

The incorporation of microfluidics-based technologies into chip design has made it possible to significantly reduce reagent volumes, leading to lower assay costs and faster results, and also to determine several targets on one platform, which can improve assay efficiency, specificity and sensitivity and thus ultimately might lead to better mastitis treatment. In theory, all these assays could be carried out 'on-site', thus providing a rapid mastitis detection format.

Conclusions

Continuous monitoring of mastitis, and its careful management, is essential for the well-being of a dairy herd. This can be achieved through the detection of inflammation at its early stages and, subsequently, the detection and treatment of the mastitis infection. Traditional and well-established tests include SCCs and culture-based methods. Assays mainly used 'on-site' are only indicative but not conclusive of the infection status of the animal. However, the development of novel analytical platforms incorporating enzymatic assays, immunoassays, biosensors and nucleic acid tests are progressively replacing the more conventional methods. Also, with advances in proteomics and genomics, new biomarkers are being discovered, allowing the disease to be detected at earlier stages. This will lead to assays with higher sensitivity, which can provide additional quantitative information on the level of inflammation 'on-site' and 'on-line' and which are also faster and less expensive. Furthermore, recent advances in microfluidics will facilitate the development of improved technologies that could subsequently be incorporated into automatic monitoring systems and portable assays for sensitive and rapid detection of mastitis.

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References

1 Yalcin, C. (2000) Cost of mastitis in Scottish dairy herds with low and high sub-clinical mastitis problems. *Turk. J. Vet. Anim. Sci.* 24, 465–472
2 Pyorala, S. (2003) Indicators of inflammation in the diagnosis of mastitis. *Vet. Res.* 34, 565–578
3 Craven, N. and Williams, M.R. (1985) Defences of the bovine mammary-gland against infection and prospects for their enhancement. *Vet. Immunol. Immunopathol.* 10, 71–127
4 Capuco, A.V. *et al.* (1992) Increased susceptibility to intramammary infection following removal of teat canal keratin. *J. Dairy Sci.* 75, 2126–2130
5 Paulrud, C.O. (2005) Basic concepts of the bovine teat canal. *Vet. Res. Commun.* 29, 215–245
6 Sordillo, L.M. and Streicher, K.L. (2002) Mammary gland immunity and mastitis susceptibility. *J. Mammary Gland Biol. Neoplasia* 7, 135–146
7 Rainard, P. and Riollet, C. (2006) Innate immunity of the bovine mammary gland. *Vet. Res.* 37, 369–400
8 Giri, S.N. *et al.* (1984) Role of prostaglandins in pathogenesis of bovine mastitis induced by *Escherichia coli* endotoxin. *Am. J. Vet. Res.* 45, 586–591
9 Paape, M.J. *et al.* (2003) The bovine neutrophil: structure and function in blood and milk. *Vet. Res.* 34, 597–627
10 Zhao, X. and Lacasse, P. (2008) Mammary tissue damage during bovine mastitis: causes and control. *J. Anim. Sci.* 86 (Suppl.), 57–65
11 Sordillo, L. and Daley, M. (1995) Role of cytokines in the prevention and treatment of bovine mastitis. In *Cytokines in Animal Health and Disease* (Myers, M.J. and Murtaugh, M.P., eds), pp. 389–419, Marcel-Dekker, Inc
12 Owen, C.A. and Campbell, E.J. (1999) The cell biology of leukocyte-mediated proteolysis. *J. Leukoc. Biol.* 65, 137–150
13 Bank, U. and Ansorge, S. (2001) More than destructive: neutrophil-derived serine proteases in cytokine bioactivity control. *J. Leukoc. Biol.* 69, 197–206
14 Paape, M. *et al.* (2002) Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. *J. Mammary Gland Biol. Neoplasia* 7, 109–121
15 Lee, C.S. *et al.* (1980) Identification, properties, and differential counts of cell populations using electron microscopy of dry cows secretions, colostrum and milk from normal cows. *J. Dairy Res.* 47, 39–50
16 Kitchen, B.J. (1981) Review of the progress of dairy science – bovine mastitis – milk compositional changes and related diagnostic-tests. *J. Dairy Res.* 48, 167–188
17 Milner, P. *et al.* (1996) Detection of clinical mastitis by changes in electrical conductivity of foremilk before visible changes in milk. *J. Dairy Sci.* 79, 83–86
18 Schukken, Y.H. *et al.* (2003) Monitoring udder health and milk quality using somatic cell counts. *Vet. Res.* 34, 579–596
19 Schalm, O.W. and Noorlander, D.O. (1957) Experiments and observations leading to development of the California mastitis test. *J. Am. Vet. Med. Assoc.* 130, 199–204
20 Lippolis, J.D. and Reinhardt, T.A. (2005) Proteomic survey of bovine neutrophils. *Vet. Immunol. Immunopathol.* 103, 53–65
21 van Leeuwen, W.B. *et al.* (2005) Host- and tissue-specific pathogenic traits of *Staphylococcus aureus*. *J. Bacteriol.* 187, 4584–4591
22 Smolenski, G. *et al.* (2007) Characterisation of host defence proteins in milk using a proteomic approach. *J. Proteome Res.* 6, 207–215
23 Baeker, R. *et al.* (2002) Lipocalin-type prostaglandin D synthase in milk: a new biomarker for bovine mastitis. *Prostaglandins Other Lipid Mediat.* 67, 75–88
24 Hogarth, C.J. *et al.* (2004) Differential protein composition of bovine whey: a comparison of whey from healthy animals and from those with clinical mastitis. *Proteomics* 4, 2094–2100
25 Yang, Y. *et al.* (2009) Proteomic analysis of mammary tissues from healthy cows and clinical mastitic cows for identification of disease-related proteins. *Vet. Res. Commun.* 33, 295–303
26 Taverna, F. *et al.* (2007) Characterization of cell wall associated proteins of a *Staphylococcus aureus* isolated from bovine mastitis case by a proteomic approach. *Vet. Microbiol.* 119, 240–247
27 Radostits, O.M. *et al.*, eds (2007) *Veterinary Medicine* (10th edn), pp. 673–762, Saunders Elsevier
28 Fox, L.K. and Adams, D.S. (2000) The ability of the enzyme-linked immunosorbent assay to detect antibody against *Staphylococcus aureus* in milk following experimental intramammary infection. *J. Vet. Med. B. Infect. Dis. Vet. Public Health* 47, 517–526
29 Kalorey, D.R. *et al.* (2007) Evaluation of indirect and avidin-biotin enzyme-linked immunosorbent assays for detection of anti-listeriolysin O antibodies in bovine milk samples. *Zoonoses Public Health* 54, 301–306
30 Yazdankhah, S.P. *et al.* (1998) Rapid and sensitive detection of *Staphylococcus* species in milk by ELISA based on monodisperse magnetic particles. *Vet. Microbiol.* 62, 17–26
31 Iannelli, D. *et al.* (1998) Simultaneous identification of antibodies to *Brucella abortus* and *Staphylococcus aureus* in milk samples by flow cytometry. *J. Clin. Microbiol.* 36, 802–806

32 Barbuddhe, S.B. *et al.* (2002) The occurrence of pathogenic *Listeria monocytogenes* and antibodies against listeriolysin-O in buffaloes. *J. Vet. Med. B. Infect. Dis. Vet. Public Health* 49, 181–184
33 Arimi, S.M. *et al.* (2005) Risk of infection with *Brucella abortus* and *Escherichia coli* O157:H7 associated with marketing of unpasteurized milk in Kenya. *Acta Trop.* 96, 1–8
34 Arora, S. *et al.* (2006) Comparison of ELISA and PCR vis-à-vis cultural methods for detecting *Aeromonas* spp. in foods of animal origin. *Int. J. Food Microbiol.* 106, 177–183
35 Vaidya, V.M. *et al.* (2008) Prevalence of Q fever in domestic animals with reproductive disorders. *Comp. Immunol. Microbiol. Infect. Dis*, DOI: 10.1016/j.cimid.2008.10.006
36 Gronlund, U. *et al.* (2003) Haptoglobin and serum amyloid A in milk and serum during acute and chronic experimentally induced *Staphylococcus aureus* mastitis. *J. Dairy Res.* 70, 379–386
37 Hiss, S. *et al.* (2004) Haptoglobin concentrations in blood and milk after endotoxin challenge and quantification of mammary Hp mRNA expression. *J. Dairy Sci.* 87, 3778–3784
38 Eckersall, P.D. (2007) Acute phase protein: biomarkers of disease in cattle and sheep. *Cattle Practice* 15, 240–243
39 Åkerstedt, M. *et al.* (2008) Relationship between haptoglobin and serum amyloid A in milk and milk quality. *Int. Dairy J.* 18, 669–674
40 Molenaar, A.J. *et al.* (2009) The acute-phase protein serum amyloid A3 is expressed in the bovine mammary gland and plays a role in host defence. *Biomarkers* 14, 26–37
41 Szczubial, M. *et al.* (2008) Concentration of serum amyloid A and activity of ceruloplasmin in milk from cows with clinical and sub-clinical mastitis. *Bull. Vet. Inst. Pulawy* 52, 391–395
42 Studer, E. *et al.* (2008) A longitudinal field study to evaluate the diagnostic properties of a quantitative real-time polymerase chain reaction-based assay to detect *Staphylococcus aureus* in milk. *J. Dairy Sci.* 91, 1893–1902
43 Cremonesi, P. *et al.* (2005) Development of a multiplex PCR assay for the identification of *Staphylococcus aureus* enterotoxigenic strains isolated from milk and dairy products. *Mol. Cell. Probes* 19, 299–305
44 Phuektes, P. *et al.* (2003) Multiplex polymerase chain reaction as a mastitis screening test for *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae* and *Streptococcus uberis* in bulk milk samples. *J. Dairy Res* 70, 149–155
45 Gillespie, B.E. and Oliver, S.P. (2005) Simultaneous detection of mastitis pathogens, *Staphylococcus aureus, Streptococcus uberis*, and *Streptococcus agalactiae* by multiplex real-time polymerase chain reaction. *J. Dairy Sci.* 88, 3510–3518
46 Cai, H.Y. *et al.* (2005) Development of a real-time PCR for detection of *Mycoplasma bovis* in bovine milk and lung samples. *J. Vet. Diagn. Invest.* 17, 537–545
47 Glynn, B. *et al.* (2006) Current and emerging molecular diagnostic technologies applicable to bacterial food safety. *Int. J. Dairy Technol.* 59, 126–139
48 O'Grady, J. *et al.* (2008) Rapid real-time PCR detection of *Listeria monocytogenes* in enriched food samples based on the *ssrA* gene, a novel diagnostic target. *Food Microbiol.* 25, 75–84
49 Koskinen, M.T. *et al.* (2009) Analytical specificity and sensitivity of a real-time polymerase chain reaction assay for identification of bovine mastitis pathogens. *J. Dairy Sci.* 92, 952–959
50 Gore, H.M. *et al.* (2003) Real-time molecular beacon NASBA reveals *hblC* expression from *Bacillus* spp. in milk. *Biochem. Biophys. Res. Commun.* 311, 386–390
51 Hovinen, M. *et al.* (2008) Detection of clinical mastitis with the help of a thermal camera. *J. Dairy Sci.* 91, 4592–4598
52 Colak, A. *et al.* (2008) Short communication: early detection of mastitis using infrared thermography in dairy cows. *J. Dairy Sci.* 91, 4244–4248
53 Hiss, S. *et al.* (2007) Haptoglobin and lactate dehydrogenase measurements in milk for the identification of sub-clinically diseased udder quarters. *Vet. Med.* 52, 245–252
54 Frundzhyan, V.G. *et al.* (2008) Improved bioluminescent assay of somatic cell counts in raw milk. *J. Dairy Res.* 75, 279–283
55 Kamphuis, C. *et al.* (2008) Using sensor data patterns from an automatic milking system to develop predictive variables for classifying clinical mastitis and abnormal milk. *Comput. Electron. Agric.* 62, 169–181
56 Hovinen, M. *et al.* (2006) Accuracy and reliability of mastitis detection with electrical conductivity and milk colour measurement in automatic milking. *Acta Agric. Scand. A-An.* 56, 121–127
57 Norberg, E. (2005) Electrical conductivity of milk as a phenotypic and genetic indicator of bovine mastitis: a review. *Livest. Prod. Sci.* 96, 129–139
58 Rasmussen, M.D. and Bjerring, M. (2005) Visual scoring of milk mixed with blood. *J. Dairy Res.* 72, 257–263
59 Mottram, T. *et al.* (2007) Evaluation of a novel chemical sensor system to detect clinical mastitis in bovine milk. *Biosens. Bioelectron.* 22, 2689–2693
60 Eriksson, A. *et al.* (2005) Detection of mastitic milk using a gas-sensor array system (electronic nose). *Int. Dairy J.* 15, 1193–1201
61 Hettinga, K.A. *et al.* (2008) Detection of mastitis pathogens by analysis of volatile bacterial metabolites. *J. Dairy Sci.* 91, 3834–3839
62 Davis, S.R. *et al.* (2004) Milk L-lactate concentration is increased during mastitis. *J. Dairy Res.* 71, 175–181
63 Davis, S. *et al.* (2000) New Zealand Pastoral Agriculture Research Institute Limited. Mastitis detection method, European Patent no. EP1192460
64 Pemberton, R.M. *et al.* (2001) An assay for the enzyme N-acetyl-β-D-glucosaminidase (NAGase) based on electrochemical detection using screen-printed carbon electrodes (SPCEs). *Analyst (Lond.)* 126, 1866–1871
65 Day, W. (2005) Engineering precision into variable biological systems. *Ann. Appl. Biol.* 146, 155–162
66 Akerstedt, M. *et al.* (2006) Biosensor assay for determination of haptoglobin in bovine milk. *J. Dairy Res.* 73, 299–305
67 Whyte, D. *et al.* (2005) Chemical and rheological aspects of gel formation in the California mastitis test. *J. Dairy Res.* 72, 115–121
68 Wu, J.Y. *et al.* (2005) Deoxyribonucleic acid sensor for the detection of somatic cells in bovine milk. *Biosyst. Eng.* 90, 143–151
69 Garcia-Cordero, J.L. and Ricco, A.J. (2008) Lab on a chip (general philosophy). In *Encyclopedia of Micro- and Nanofluidics* (Dongqing, L., ed.), pp. 962–969, Springer
70 Moon, J.S. *et al.* (2007) Application of a new portable microscopic somatic cell counter with disposable plastic chip for milk analysis. *J. Dairy Sci.* 90, 2253–2259
71 Rodriguez, R.R. and Galanaugh, C.F. (2007) Advanced animal diagnostics. Microfluidic chamber assembly for mastitis assay, PCT Patent no. WO/2007/112332
72 Garcia-Cordero, J.L. and Ricco, A.J. (2008) A milk analysis microfluidic apparatus for detecting mastitis in a milk sample by isolating somatic cells suspended therein in the form of pellets of cells using centrifugal sedimentation, British Patent Application No. GB0801991.1
73 Choi, J.W. *et al.* (2006) Lab-on-a-chip for monitoring the quality of raw milk. *J. Microbiol. Biotechnol.* 16, 1229–1235
74 Lee, K.H. *et al.* (2008) Development of a novel biochip for rapid multiplex detection of seven mastitis-causing pathogens in bovine milk samples. *J. Vet. Diagn. Invest.* 20, 463–471
75 Dimov, I.K. *et al.* (2008) Integrated microfluidic tmRNA purification and real-time NASBA device for molecular diagnostics. *Lab Chip* 8, 2071–2078
76 Hillerton, J.E. and Berry, E.A. (2005) Treating mastitis in the cow – a tradition or an archaism. *J. Appl. Microbiol.* 98, 1250–1255
77 Donovan, D.M. *et al.* (2005) Engineering disease resistant cattle. *Transgenic Res.* 14, 563–567
78 Huijps, K. *et al.* (2008) Costs of mastitis: facts and perception. *J. Dairy Res.* 75, 113–120