Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 July 2022 doi:10.20944/preprints202207.0200.v1

Review

miRNA Sensing in Livestock: challenges and potential approaches

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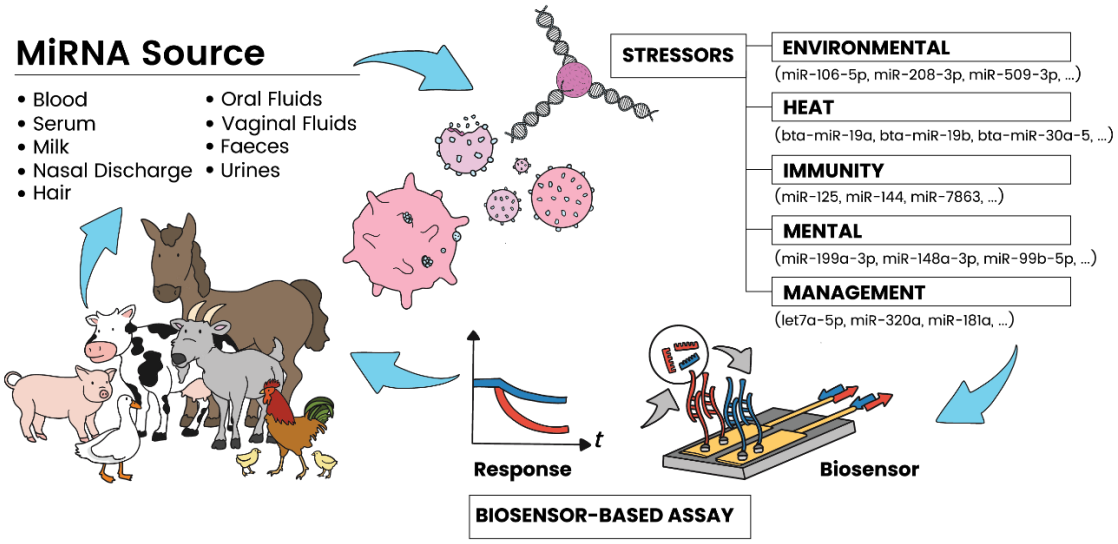

Simple Summary: Early disease detection in livestock allows for target treatment decreasing antibiotics use and allow advancements in precision veterinary medicine. MicroRNA (miRNA) -driven signaling cascades play a crucial role in the context of farm animal disease diagnostics and prediction, and their proper understanding remains a challenge. In livestock farm animals, only a small number of miRNAs have been fully validated with respect to disease conditions and physiological or behavioral traits. Low abundance of miRNAs in blood and bodily fluids, along with a small number of nucleotides, makes detection and discrimination tedious and challenging task in. miRNAs usually are homologous, owing to which detection specificity becomes next to impossible when screening for multiple miRNAs in the same analyte sample. Hence, a concurrent, multiplexing, approach becomes crucial for the development of on-farm point-of-care based detection systems. Comprehensive screening methods demand broad dynamic range and enhanced specificity. For on-farm handheld platform development, the ability to screen for multiple varieties of miRNA is essential. In this review paper, I provide an overview of the recent developments of miRNA sensing and the current bottlenecks in the realization of the sensors for detecting miRNAS as target analyte for various livestock disease detection applications. Due to the nascent stages of this research, the possibilities of exploiting miRNAs as a biomarker opens up ways to move from reactive to predictive possibilities in diseases detection in the modern digital livestock farming.

Abstract: The versatility of livestock makes them central to human nutrition. Globally, the livestock sector represents a $1.4 trillion market employing at least 1.3 billion people. However, the farming of animals for food raises complex questions regarding livestock diseases and their potential impacts on both human health along with national and international export trade markets. Circulating miRNAs may provide useful biomarkers of disease in livestock. Many studies have shown a link between circulating miRNAs and diseases in livestock, including paratuberculosis, foot and mouth disease, and various metabolic diseases. Important information regarding the stage of a disease, pathogenesis, and prognosis may therefore be determined thorough the detection and analysis of a

small number of miRNAs. Given the rapidly increasing demand for livestock products, perhaps the biggest challenges that face the livestock industry today are addressing the health, growth, and reproduction of animals. While developments in animal breeding, nutrition, and veterinary care will continue to contribute to increasing potential production and commercial efficiency, significant challenges remain. These include the need to address and implement approaches for the accurate sensing, diagnosis, and treatment of animal diseases. This review sought to establish the potential of circulating miRNAs as biomarkers of disease in livestock and the current state of technology for their detection.

Keywords: microRNAs; Precision livestock science; animal welfare; livestock health; biomarkers; biosensor; pandemics

1. MicroRNAs: Small molecules, high impact

The past two decades have seen a revolution in the development of rapid, low-cost, and high-specificity molecular biology techniques for characterizing and quantitating DNA, RNA, and proteins. Within cells, numerous species of RNA molecules exist. These include messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). RNAs can be further classified as either coding (cRNA) or non-coding RNA (ncRNA) [1]. Two types of ncRNAs exist - housekeeping ncRNAs (tRNA and rRNA) and regulatory ncRNAs. ncRNAs are typically classified according to their size. Long ncRNAs (lncRNAs) are at least 200 nucleotides long. In contrast, ncRNAs have fewer than 200 nucleotides. MicroRNAs (miRNA) are a species of regulatory ncRNAs (Glossary). Others in this category include small interference RNAs (siRNAs), long non-coding RNAs (lncRNAs), circular RNAs (circRNAs), piwi-associated RNAs (piRNAs) and tRNAs [2]. Most recently, there has been significant interest surrounding the role of non-coding RNAs as active modulators of protein-coding gene function. MicroRNAs (miRNAs) comprise a family of endogenously expressed, small (~22 nucleotides in length), non-coding transcripts. The binding of miRNAs to complementary sequences in the 3′-untranslated regions of target mRNAs results in the posttranscriptional regulation of both gene expression and protein translation via the inhibition of translation initiation and elongation [3]. miRNAs are known to regulate the expression of genes involved in various biological processes, including signal transduction [4], cell cycle [5], differentiation [6], proliferation [7], and apoptosis [8]. Endogenous mRNAs contain multiple target sites for several miRNAs [9]. miRNAs are able to regulate most protein-coding transcripts with the most recent estimates suggesting that at least 60% of gene expression is regulated by miRNAs [10]). In some cases, miRNAs are known to target a large number (100-200) of different mRNAs [11]. Endogenous transcription factors are known to be able to repress and enhance the expression of miRNA depending on the availability of a particular factor [12]. In the context of disease states, miRNA genetic variability has been hypothesized to play a role in the susceptibility of livestock to diseases [13].

Role of miRNAs in Livestock Diseases and Conditions:

Extracellular miRNAs have been detected in the peripheral blood of a number of animal species, including bovines [14], poultry [15], and pigs [16]. As a result of their high stability in the body fluids of livestock, there is significant interest in the use of circulating miRNA as biomarkers for both the detection and monitoring of disease progression in animals [17] [18]. Expression-profiling studies conducted in domestic livestock have determined that miRNAs have distinct spatial (e.g., cell and tissue) and temporal expression patterns [19]. The aberrant expression of miRNAs can impair the regulation of many cellular functions and gene networks. Studies have shown a link between

 doi:10.20944/preprints202207.0200.v1

circulating miRNAs and diseases in livestock, including paratuberculosis [20], foot and mouth disease [21], and various metabolic diseases [22]. As such, important information regarding the stage of a disease, pathogenesis, and prognosis may be determined thorough the detection and analysis of a small number of miRNAs. miRNAs have been detected in several bodily fluids of animals, including plasma, serum, and milk. Typically, miRNAs are released into extracellular environments in protein complexes or within microvesicles or exosomes [23]. There is increasing evidence that specific miRNAs are altered in various disease states in livestock [24]. Similarly, for a large number of livestock diseases, predicting the health status, resilience, and even for mental functions, miRNAs have been found to play important roles. In response to various pathogens, including mycoplasma bovis, bovine viral diarrhea virus, and Staphylococcus aureus, miRNAs with differential expression have been reported in cattle.

Immunity. By regulating the differentiation and function of immune cells, a role for miRNAs in bovine immunity has been proposed [25]. miRNAs have been hypothesized to play a crucial role in bovine immunity by controlling immune cell differentiation and function. The differential expression of miRNAs has been reported in bovines in response to infection by pathogens. These include mycoplasma bovis, bovine viral diarrhea virus, and Staphylococcus aureus [26] [27] [28].

Mycobacterium avium ssp. paratuberculosis. Changes in the levels of circulating miRNAs have been identified in the serum [20], whole blood [29] and ileal tissues [30] of cattle infected with Mycobacterium avium ssp. paratuberculosis (MAP). MAP-infected cattle display progressive granulomatous inflammation of the ileum giving rise to a condition known as Johne's Disease. This disease causes diarrhea, weight loss, and even death in animals and results in considerable losses to the agriculture industry worldwide every year.

Foot and mouth disease. This is a highly contagious viral disease that affects cloven-hoofed animals (those with divided hoofs), including cattle, sheep, goats, buffalo, deer, pigs, and camels.

Heat stress

The detection of specific miRNAs in the blood of cows is an important tool for assessing the responses of animals to heat stress. Elevated environmental temperatures can negatively affect the health and productivity of animals. In a study by Lee et. al., [31] blood samples from cows were collected to check the expression of both mRNA and miRNA in different environmental conditions. A total of 11 miRNAs (bta-miR-19a, bta-miR-19b, bta-miR-30a-5p, and several from the bta-miR-2284 family) were differentially expressed in both pregnant and non-pregnant cows under heat stress conditions. Another study of the effect of heat stress on pregnant cows showed that the miRNAs bta-miR-146b, bta-miR-20b, bta-miR-29d-3p, and bta-miR-1246 that specifically target progesterone biosynthesis (StAR) and the function of corpus luteum-related genes (CCL11, XCL) were differentially expressed [31]. Similarly, a study by Li et al., [32] showed in heat-stressed Holstein cattle, 20 miRNAs had higher levels of expression in their mammary tissues. The seven highest differentially expressed candidate miRNAs were bta-miR-21-5p, bta-miR-99a-5p, bta-miR-146b, bta-miR-145, bta-miR-2285 t, bta-miR-133a, and bta-miR-29c.

Tumorigenesis and Cancer

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Recent studies have highlighted the important role that abnormal miRNA expression plays in the pathogenesis of tumorigenesis and in various cancers [33]. In livestock, cancer is a highly problematic disease owing to its high rate of mortality and expensive treatment costs, which often have only limited success. The earlier detection of tumors and the estimation of correct subtypes can significantly aid in the determination of appropriate treatment regimens that can aid in lowering the time and incurred risks for treating infected animals [34]. Additionally, if appropriate treatment is started before the disease reaches an advanced stage, the cost of risk can be curtailed. miRNAs may therefore offer significant diagnostic value for detecting and possibly predicting certain cancers.

Pregnancy and Lactation

In bovines, miRNAs are known to be present in the mammary glands; however, they are not present in the glands of animals who are either non-pregnant or non-lactating [35]. In milk, miRNAs are present in exosomes and are released into the body fluids by different types of cells. miRNAs contribute to the regulation of follicular and luteal development along with the endometrial function. In the mammary glands themselves, miRNAs play important roles in several cellular processes. The expression of miR-10a, miR-15b, miR-16, miR-21, miR-31, miR-33b, miR-145, miR-146b, miR-155, miR-181a, miR-205, miR-221, and miR-223 has been studied at periods −30 days prepartum, 7 days postpartum, and 30 days postpartum [36]. When analyzed, all miRNAs apart from miR-31 showed increases in expression between −30 days prepartum and 7 days postpartum. Similarly, the expression of miR-221 was shown to further increase 30 days postpartum, which corresponded to early lactation. As a result, a role for miR-221 in the control of endothelial cell proliferation and/or angiogenesis was proposed. In contrast, miR-223 was found to be decreased at early lactation, which suggests that they may play a role in the mammary response to pathogens in the periods following parturition. miR-31, a known inhibitor of cyclin gene expression and which is hormonally regulated, was found to be expressed at higher levels at timepoints corresponding to early lactation when compared to −30 days prepartum. Similarly, the upregulation of miR-33b during early lactation may play a role in lipogenesis in mammary tissue. The presence of miRNAs in cattle milk also depicts the role of miRNAs in the gastrointestinal system and for their potential role in modulating the immune system. A study by Ye et. al. [37] showed that one of the most critical miRNAs, microRNA-145 (mir-145), is a potent tumor suppressor that regulates multiple cellular pathways, including those involved in regulating heat stress in mammary tissues. MicroRNA-145 has been shown to be down-regulated in many types of cancer. It regulates several cellular processes, including entry into the cell cycle, proliferation, and apoptosis. Additionally, mir-145 has been shown to play a critical role in cellular invasion through the targeting of multiple oncogenes [38].

Using small RNA sequencing and RT-qPCR, Ioannidis et al. [39] were able to identify 92 miRNAs that showed significantly higher expression in plasma compared with paired blood cell samples (n = 4 cows). Interestingly, three miRNAs – miR-122 (liver), miR-133a (muscle) and miR-215 (intestine) were found to be enriched in tissues, while miR-802 which reportedly regulates insulin sensitivity and lipid metabolism, a key parameter in the context of post-partum negative energy balance in dairy cows, was highly enriched specifically in the liver (Table 1).

Another significant role of miRNAs in livestock is that they accommodate pregnancy diagnosis, most notably acting as a biomarker for early pregnancy detection. Circulating miRNA signatures of early pregnancy in cattle have been determined. MiRNAs have been shown to regulate various biological functions, including ovarian function, uterine receptivity, embryonic development, and placental function (Table 1) [40].

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Endometritis. This is a significant reproductive disorder in dairy cattle. The disorder tends to result in reduced fertility and milk production. The endometrium of cows serves to act as a barrier to the pathogen invasion of uterine tissues. By controlling the inflammatory immune response, endometrial epithelial cells are able to mount a response for resisting pathogen invasion [41]. miR-21-3p, has been suggested to play an important role in promoting the viability and proliferation of the epithelial tissue in the mammary glands of dairy cows [42].

Challenges to miRNA detection

The on-field detection of miRNA for disease detection is vital to ensuring not only the future safety of our food supply but the economic viability of farmers across the world. In light of the recent emergence of new epidemic and pandemic-causing diseases, the need for tools for rapidly identifying infections will be crucial in halting cross-species disease transmission. It is therefore important, that to understand the role that miRNA plays in the pathogenesis of disease that tools are available for detecting the levels of expression of these molecules. However, significant challenges remain in the reliable detection of miRNA from blood and other bodily fluids of livestock as a result of their small size, low abundance in samples, and high sequence similarity. A critical factor is their susceptibility to degradation.

miRNA research: Technological advancements

Since the discovery of miRNAs, research into their detection and characterization has given rise to a rather exciting field. The latest release of miRBase (version 22) [43] provides data for 38,589 pre-miRNAs from 271 organisms, including livestock. To gain a better understanding of their function and fully understand the complete repertoire of their biological roles, new tools are required. Traditional methods for miRNA detection include quantitative polymerase chain reaction (qPCR) [44], microarray analysis [45], and northern blotting [46]. However, these methods for miRNA detection and analysis are not capable of meeting the current needs. These include the relatively high cost for equipment and reagents, time-consuming protocols, limited use among input samples (e.g., blood versus feces), and low detection sensitivity.

Next-generation sequencing (NGS). NGS is a valuable tool for investigating the complexity of the miRNA transcriptome (Glossary). NGS is a novel method widely used in animal science and veterinary research [47]. The use of high-throughput NGS technologies in livestock research has enabled not only developments in metagenomics and genome-wide association studies but also an elucidation of the role of miRNAs in livestock health. A novel NGS approach was used to profile the expression of miRNAs in primary bovine mammary epithelial cells following infection with Streptococcus uberis, a causative agent of bovine mastitis [48]. An analysis of over 450 million sequencing reads identified 21 miRNAs that were identified as significantly differentially expressed post-infection with S. uberis. For example, the miRNA bta-let-7 was up-regulated at early time-points post-infection (4- and 6-hours). The let-7 family is known to play a role in the immune response.

RNA sequencing (RNA-Seq). This is one of the major technology advancements in the study of ncRNA species, such as miRNA. RNA-Seq offers researchers the ability to identify change(s) in the levels of expression of RNA species that cannot currently be performed using current PCR, microarray-, or Northern blotting-based platforms. However, RNA-Seq is costly and requires specialized software and hardware requirements (i.e., because of intensive computing power). Importantly, RNA-Seq is capable of identifying transcript variants of miRNA or miRNA isomers ("isomiRs"). IsomiRs are responsible for

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the large diversity of miRNA sequence variants ("isomiRs"). These result from the processing or post-transcriptional modification to miRNAs. For example, changes to the seed sequence (nucleotides 2–8), including shifted start positions of a miRNA, can result in the redirection of targeting to a different set of target RNAs.

MinION and GridION. These related technologies developed by Oxford Nanopore Technologies for the direct electronic analysis of DNA, RNA, proteins, and single molecules MinION determined the identify of DNA bases by measuring the changes in electrical conductivity generated as DNA strands pass through a biological pore [49] (Glossary). GridION is a desktop device that allows simple scaling of MinION and Flongle Flow Cells. Using GridION, users can simultaneously run up to five flow cells, enabling the generation of as much as 150Gb sequence data in a single run. These tools offer applications for portable sensing and were extensively used in field diagnosis in the African swine fever outbreak in China in 2020 [50]. A future application of this technology is "crush-side genotyping," which has been specifically designed for the real-time, on-farm genotyping of livestock. MinION sequencing has been used to functionally characterize S. suis bacteria in pigs. These bacteria are important pathogens for pigs and have recently developed antimicrobial resistance. Whole genome generation and characterization profiling, including miRNA sequences were successfully generated using the MinION technology.

Ion Torrent sequencing. A different approach to NGS, released in 2010, was developed by Ion Torrent Systems, Inc. The protocol for Ion Torrent technology is simple, requiring only a simple, four-step workflow: library construction, template preparation, sequencing, and analysis. Ion Torrent sequencing was the first commercial sequencing technology not based on dye-labeled oligonucleotides nor requiring expensive optics. Instead, it uses measurements of H+ ions released during base incorporation, and it is uniquely suited for amplicon sequencing. The sequencing method has been used to identify differentially expressed microRNAs in the Sahiwal (Bos indicus) [50] and Frieswal (Bos taurus x Bos indicus) breeds of cattle during heat stress [51]. In the latter study, out of a total of 420 miRNAs investigated, most were found to target heat shock responsive genes, especially members of the heat shock protein (HSP) family. Of the 420, a total of 65 were differentially expressed during peak summer temperatures, including bta-miR-2898, which is known to target HSPB8 (heat shock protein 22) (51). Ion sequencing has also been used to determine the profiles of expression of host and viral miRNAs in Aujeszky's disease virus [ADV] (also known as suid herpesvirus type 1 [SuHV-1]) in pigs [52]. Data showed that miR-206, miR-133a, miR-133b, and miR-378 were differentially expressed between animals that were infected with the virus.

Electrochemical Sensing. Approaches to electrochemical sensing include amperometric and voltammetric-, potentiometric-, conductometric-, impedimetric-, and field-effect transistor-based biosensors [53]. Amperometry-based sensors detect analytes by measuring the current of constant applied potential. Measurement of the current is then related to the concentration of the target analyte. In contrast, voltametric measurements measure the current as the potential is raised at a given rate. Labeling and electrocatalytic amplification of miRNAs offers a promising approach to their detection. Gao et al. developed a technique for the sensing of nucleic acids with a silicon nanowire field effect transistor biosensor [54]. Using this technique, Gao et al. successfully detected miRNA in animal cells. A similar technique using hybridized miRNA-templated deposition of an insulating polymer film and electrochemical impedance spectroscopic detection was able to detect miRNA at concentrations ranging from 5.0 fM to 2.0 pM with a detection limit of 2.0 fM [55].

A similar sensor developed by Yin et al., [56] used a label-free free, electrochemical-based biosensor to detect miRNA. The detection method employed the use of dendritic gold

 doi:10.20944/preprints202207.0200.v1

nanostructures and a graphene nanosheets-modified glassy carbon electrode. Using thiol-modified locked nucleic acid (LNA) hairpin molecular beacons (MB), nucleic acids were successfully captured. The duplex formed from hybridization with the target miRNA was then probed with complementarily to the distal terminus of the capture probe. Modification with biotin coated gold nanoparticles and detection using streptavidin-modified horseradish peroxidase, permitted the chemical oxidation of hydroquinone to benzoquinone following the addition of hydrogen peroxide. The biosensor as able to detect miRNAs down to 0.06 pM.

Loop-mediated isothermal amplification (LAMP). LAMP techniques offer the advantages of being able to be performed without the need for precise control of temperature cycling (compared with PCR) [57] [58]. The technique is well suited for detecting short RNA sequences, such as miRNAs [59]. As a result of the relatively mild reaction conditions, LAMP enables the detection of miRNA in single cells, which can be vital to determining the biological reactions to infection in lineage-specific cells. Ge et al. used a novel and ultrasensitive detection platform for microRNA detection. This method works by combining tetrahedral-structured, DNA nanostructure probes in conjunction with an HCR amplification [60]. MicroRNAs could be successfully detected at concentrations as low as 10 aM (corresponding to 600 microRNAs in a 100 μL sample). This method was able to improve the detection limits by three orders of magnitude when compared with the widely used super-sandwich amplification [61]. SDA assays allow for exponential amplification of miRNA. The process is miRNA-initiated through a Klenow fragment polymerase, a nicking enzyme (such as Nt.AlwI), and two primers. During the process, the miRNA target triggers two cycles consisting of nicking, polymerization, and displacement. As a result of exponential miRNA amplification, the dsDNA products that are formed can be detected at concentrations as low as 16 zmol of the target miRNA, using SYBR Green I real-time PCR, and within 90 min [62].

A microRNA detection method that was developed by Gines et al. [63] uses isothermal amplification chemistry. This method was built around molecular programming concepts using a DNA circuit capable of converting, thresholding, amplifying, and reporting the presence of specific microRNAs within samples. The method is extremely sensitive and was capable of detecting specific miRNAs with absolute target quantification down to femtomolar concentrations. Importantly, the method can reliably suppress nonspecific amplification, that are typically encountered with other exponential amplification reactions. Most recently, the label-free detection of miRNA has been performed using surface-enhanced Raman spectroscopy (SERS). This surface-sensitive technology is based on a phenomenon that molecules boost Raman scattering adsorbed on the surface of plasmonic metals or nanostructures of plasmonic metals owing to the strong electromagnetic coupling generated in the vicinity. This technique is highly effective for the detection of nucleic acids because of its distinct benefits of high stability, strong specificity, and a low signal from the background [64]. In a SERS study by Li et al., titanium ions were used as aggregating agents that were subsequently used to induce the aggregation of silver nanoparticles. As a result of this aggregation, “hot spots” were formed allowing for fingerprint information on the miRNAs to be obtained [65].

Finally, nanobiosensors based on graphene oxide and DNA binding dye have been used in the detection of multi-miRNAs [66]. In that study, the assay was fluorescence-based and used isothermal hybridization chain reaction. SYBR Green was used as the signal, while graphene oxide was used as the fluorescence quencher. Fluorescence spectrophotometry was used for the detection and quantification of various targets miRNA. This novel graphene nano-biosensor was able to achieve a limit of detection for miRNAs from 0.05 to 5 nM – a range which is often considered to be ideal for detecting candidate biomarkers. A similar sensor involving a sandwich hybridization of a capture probe

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immobilized on a magnetic bead and a reporter probe assembled on gold nanoparticles with a miRNA target was developed by Wen et al. [67]. As a result of gold nanoparticle-catalyzed enhancement of silver staining, miRNAs were able to be detected in less than 70 min at a lower-level detection limit of 15 fM.

Surface plasmon resonance. Ultrasensitive detection of miRNAs has been accomplished using aa number of SPR techniques. A label-free, antimonene-based SPR sensor was developed by Xue et al., for miRNA detection [68]. The detection limit for miRNAs was able to reach 10 aM. Specifically, miRNA-21, whose upregulation is associated with numerous types of cancers along with miRNA-155, which plays a critical role in the immune response was able to be detected at levels 2.3–10,000 times higher than those of existing miRNA sensors. SPR has also been merged with orthogonal signal amplification to enable a direct determination of sub-fM concentrations of miRNAs [69]. Orthogonal signal amplification uses an in-plane and vertical signal amplification strategy to add additional mass on a target sample initially at the surficial direction and subsequently upwards from the surface. Using this technique, investigators were able to reach a limit of detection and limit of quantification down to 0.56 and 5fM for miRNA-15a. This represented a 107-fold improvement of sensitivity compared to other SPR methods. miRNA-15a plays an important role in gene regulation and tumor suppression.

Studies on the function of miRNAs and their interaction with genes in the modulation of macronutrients in animals showed that dietary macronutrients and the expression of specific genes and miRNA were directly related to the metabolism of these compounds (Sohel 2020). Extracellular miRNAs were found to be particularly promising markers of metabolic changes. In fact, circulating miR-935 was identified as a potential biomarker to identify individuals that responded differentially to weight loss interventions induced by energy restriction. This raises the possibility of using this type of miRNA in sensors to monitor health-related changes in the metabolism of animals based on their diets.

The potential use of miRNAs as biomarkers to evaluate the stress of livestock and improve their welfare was recently evaluated by Miretti et al. (2020). Cells actively secrete these highly stable molecules into the extracellular environment where they can be easily collected from saliva, blood, exosomes, or milk. In one study on pigs, the levels of expression of certain salivary miRNAs were more abundant in animals that had been exposed to high levels of pain, making them good candidates for potential biomarkers for pain. In another study, miR-914b-5p showed promise as a candidate biomarker for the intestinal health of piglets during weaning. Both cattle and swine are highly sensitive to heat stress, which has been correlated with the levels of other miRNA molecules. The authors stressed that accurately identifying and validating miRNAs and their target genes are essential to develop gene-based breeding strategies. Other miRNAs are upregulated following hypoxia and represent potential biomarkers for immune responses that respond to inflammatory stimuli. These studies raise the possibility that further study on these regulatory molecules will enable their use in biosensors that could be used to monitor the stress levels of livestock and therefore improve their welfare.

Given the importance of detecting miRNAs as markers of a wide range of physiological conditions, researchers have been developing new techniques to enhance the sensitivity of the methods used to detect miRNAs and measure their concentrations in bodily fluids and tissues. Current cutting-edge techniques include probes that utilize the principles of fluorescence resonance energy transfer (FRET) and bioluminescent resonance energy transfer (BRET).

Progress in utilizing BRET techniques was elucidated by Li et al. (2019), who developed a paper-based system that was highly stable at room temperature and could detect femtomolar concentrations of miRNAs. Chen et al (2020) and Wang et al (2019) reviewed

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developments in the use of new fluorescent hybridization probes to improve the sensitivity of existing probes. Using nucleic acid probes coupled with gold nanoparticles, miRNAs could be detected at concentrations of 1.5 femtomolars using the FRET technique. The ability to measure such low concentrations enables the detection of trace amounts of these molecules in real samples and can also distinguish homologous miRNAs. These results bode well for the future use of these techniques in biosensors to quantify key miRNAs that are associated with the effects of stress on animals.

Table 1. MiRNAs as indicators of physiological, behavioural and biological functions of livestock.

Livestock Disease/Condition	Analyte source	miRNAs involved	miRNA characteristics	Detection method	References
Pregnancy					
Pregnancy diagnosis	Plasma samples	miR-99b, miR-152, miR-101, miR-103	C19MC cluster miRNAs, miR-516-5p, miR-518b, miR-520a and miR-525	Small-RNA sequencing, RT-qPCR	[39]
Pregnancy and lactation in Holstein cows	Mammary gland tissue, serum	Group 1. miR-10a, miR-15b, miR-16, miR-21, miR-31, miR-33b, miR-145, miR-146b, miR-155, miR-181a, miR-205, miR-221, and miR-223	Group 1. All miRNAs except miR-31 showed increases in expression between −30 days prepartum and 7 days postpartum miR-31, a known inhibitor of cyclin gene expression Expression of miR-221 was shown to further increase 30 days postpartum which corresponded to early lactation. A role for miR-221 in the control of endothelial cell proliferation	RT-qPCR	[36]

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			and/or angiogenesis		
Retained fetal membrane syndrome	Serum	miR-185	miRNA-185 regulates the VEGFA signaling pathway in dairy cows with retained fetal membranes	qPCR and Western blotting	[70]
Endometritis	Bovine mammary gland epithelial cells	miR-21-3p	Plays an important role in promoting the viability and proliferation of the epithelial tissue in the mammary glands of dairy cows	MTT assay, flow cytometry analysis, Dual luciferase assay, RT-qPCR, and Western blot	[41]
Liver function and post-partum	Blood cells, plasma	miR-802	Insulin sensitivity and lipid metabolism	Small RNA sequencing, RT-qPCR	[38]
Heat stress in pregnancy	Serum	Group 1. bta-miR-19a, bta-miR-19b, bta-miR-30a-5p, bta-miR-2284 Group 2. bta-miR-146b, bta-miR-20b, bta-miR-29d-3p, bta-miR-1246	Group 1. miRNAs were differentially expressed in both pregnant and non-pregnant cows under heat stress conditions. Group 2. targeted progesterone biosynthesis (StAR) and the function of corpus luteum-related genes (CCL11, XCL),		[31]
Heat stress					
Heat stress	Serum	bta-miR-21-5p, bta-miR-99a-5p, bta-miR-146b, bta-miR-145, bta-miR-2285, bta-	miRNAs may act as dominant regulators during heat stress	Deep RNA sequencing, stem-loop qPCR	[32]

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		miR-133a, bta-miR-29c bta-miR-423-5p			
Diseases					
Paratuberculosis	Serum	1. miR-1976, miR-873-3p, miR-520f-3p, and miR-126-3p 2. Increase in miR-6517, miR-7857, miR-24-1, miR-24- 2, miR-378c 3. Decrease in miR-19b, miR-19b-2, miR-1271, miR100, miR-301a, miR-32a	miRNA expression can distinguish between moderate and severely infected animals from noninfected animals.	NanoString nCounter technology	[20]
Foot and mouth disease	Serum	bta-miR-21-5p, bta-miR-101, bta-miR-126-3p, bta-miR-145, bta-miR-197, bta-miR-223	Compared to prior to infection, on day 2 post-infection, 119 miRNAs were upregulated, of which 39 were significantly upregulated. serum miRNA upregulation before the appearance of clinical signs. These circulating miRNAs, which are released by lysed cells or secreted from cells in a paracrine manner	Microarray, RT-qPCR	[21]

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Foot and mouth disease	Serum	Group 1. bta-miR-23b-5p, let-7 g, bta-miR-22-5p, bta-miR-1224, bta-miR-144, bta-miR-497, bta-miR-455-3p, bta-miR-154a, bta-miR-369-3p, bta-miR-26b, bta-miR-34a, bta-miR-205, bta-miR-181b, bta-miR-146a, bta-miR-17-5p, bta-miR-31 Group 2. bta-miR-26b, bta-miR-34a, bta-miR-205, bta-miR-181b, bta-miR-146a, bta-miR-17-5p, bta-miR-31, bta-miR-150, bta-miR-147 3. miR-1281, bta-miR-17-5p, bta-mir-31	Group 1. Cellular proliferation or apoptosis Group 2. Immune modulatory function Group 3. Tumour suppressors	miRNA PCR array plates	[71]
Bovine mastitis	Milk-isolated monocytes	bta-miR-615, bta-miR-451, bta-miR-451, bta-miR-146b, bta-miR-411a, miR-149	Downregulated miRNAs are highly enriched for roles in innate immunity; upregulated miRNAs preferentially target genes	Next-generation sequencing	[25]

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			involved in metabolism		
Mycoplasma bovis	Serum	1. bta-let-7b, bta-miR- 24-3p, bta-miR- 92a, bta-miR-423-5p 2. Increase miR-155. miR-146a, miR-146b-5p, miR-886-5p 3. Decrease miR-20a, miR-191, miR-378, miR-30c, miR-423-5p. miR-374a, miR-185, miR768-5p, miR-18	These microRNAs have been recognized as playing a role in the host defense against bacteria.	Next generation sequencing	[26]
Bovine viral diarrhea virus	Serum	Bta-miR-423-5p, bta-miR-151-3p	miRNA expression involved in host immune response	Next generation sequencing	[27]
Staphylococcus aureus	Bovine milk exosomes	bta-miR-142-5p, bta-miR-223	Potential biomarkers for early detection of bacterial infection of the mammary gland.	Next generation sequencing	[28]
Aujeszky's disease virus [ADV] (also known as suid herpesvirus type 1 [SuHV-1]) infection Metabolic and other disorders	Tissue samples (Olfactory bulb (OB) and trigeminal ganglia (TG)	miR-206, miR-133a, miR-133b, miR-378	Pathways related to viral infection processes and immune response	Ion Torrent sequencing, RT-qPCR	[52]
Insulin resistance			miR-1281 ction. Stenfeldt et. al., reported that was significantly decreased during		[22]

 doi:10.20944/preprints202207.0200.v1

			both the acute and chronic stages of the disease Conversely, miR-17-5p was expressed at the highest level during the acute stage. The level of bta-miR-31 was also significantly increased during the persistent stage of the disease.		
Heat stress	Serum	bta-miR-21-5p, bta-miR-99a-5p, bta-miR-146b, bta-miR-145, bta-miR-2285, bta-miR-133a, bta-miR-29c	miRNAs may act as dominant regulators during heat stress	Deep RNA sequencing, stem-loop qPCR	[32]
Acute pain, 4-d old piglets	Saliva	bta-miR-423-5p miR-19b, miR-27b-3p, miR-215, miR-22-3p, miR-155-5p, hsa-miR-365-5p, hsa-miR-204	Focal adhesion pathways, and cytokines expression	RT-qPCR	[72]
Respiratory diseases	Serum, milk	bta-miR-423-5p , bta-miR-151-3p	Avoidance of host immune response	NGS	[27]
Skeletal muscle development	Biceps femoris muscle	miR-206, mi-208b	Regulation of muscle gene expression during skeletal muscle adaptation to grazing.	RT-PCR	[73, 74]

 doi:10.20944/preprints202207.0200.v1

5. Conclusions

The livestock industry faces significant challenges in managing disease in animals. Specific types of circulating miRNAs have been correlated with specific diseases or other conditions, such as pregnancy. The accurate quantification of these molecules is paramount for their routine use in detection and can enable the early diagnosis of critical health conditions. A goal in animal care is to have biosensors that monitor the changes in hormones and miRNAs that mirror an animal's condition (Outstanding questions). Recent research has identified techniques that can identify specific miRNAs at femtomolar concentrations. These include fluorescence resonance energy transfer (FRET) and bioluminescent resonance energy transfer (BRET). Ideally, these techniques will be adapted for use in biosensors that will revolutionize the well-being of livestock and therefore the livestock industry.

Highlights

- Extracellular miRNAs have been detected in the peripheral blood of most livestock species, including cows, pigs, and poultry.
- miRNAs have distinct spatial (e.g., cell and tissue) and temporal expression patterns.
- Circulating miRNAs show significant potential as biomarkers of disease in livestock, including mycoplasma bovis, bovine viral diarrhea virus, and Staphylococcus aureus.
- Current technologies for miRNA detection include next generation sequencing, RT-qPCR, microarray, and nanopore sequencing.

Outstanding Questions Box

- miRNAs have been reported to be scarce in exosomes. Techniques need to be developed to investigate the makeup of miRNA in exosomes and distinguish exosomal cargoes in non-disease states from disease states.
- A comprehensive database of the miRNA expression profiles in different livestock tissues needs to be compiled.
- Poor and low sensitivity of sequencing technologies can lead to a relatively low number of miRNAs being identified owing to disease states.
- Circulating miRNAs play a vital role in disease pathogenesis, and an analysis of the changes in the miRNA concentrations during disease pathogenesis in livestock species remains to be determined.
- A list of microRNAs involved in different subtypes of tissues from commercial livestock species remains to be determined.
- What statistical test need to be applied to calculate the p-value for differentially expressed microRNAs in RT-qPCR data for the same species in different disease states?

Glossary

Loop-mediated isothermal amplification (LAMP): This technique uses 4-6 different primers that recognize 6-8 distinct regions of the target DNA. A strand-displacing DNA polymerase, such as phi29 or Bst DNA Polymerase, Large Fragment are capable of initiating synthesis. Two primers that have been specially designed form "loop" structures then facilitate amplification through subsequent rounds of extension on the loops and additional annealing of primers.

MicroRNAs (miRNAs): These comprise a group of small, noncoding RNA molecules, generally 21 to 24 nucleotides in length. miRNAs are normally formed via the cleavage of larger, precursor hairpin-containing RNAs. miRNAs are conserved through evolution, and their abundance and patterns of expression are suggestive of diverse regulatory roles.

MinION. This nanopore technology uses nanoscale-sized pores (1 nm-diameter) and exonuclease-based sequencing by a deconstruction approach to determine DNA sequences. The technology can be divided into two categories: biological and solid-state that correspond to pores embedded in a biological membrane or formed into a solid-state film. As DNA passes through the nanopore, the magnitude of current changes in the liquid conducting solution. For each nucleotide, the current is disturbed in a characteristic manner. This creates an electrical trace unique to each A, C, G, and T nucleotide.

Next generation sequencing (NGS): This is a massively parallel sequencing technology that possesses ultra-high throughput, scalability, and speed. The basic NGS process involves fragmenting a molecule of DNA or RNA into multiple pieces, adding adapters, and then sequencing every molecule. Computers are then used to reassemble the sequences to construct a library of genomic sequences. NGS utilizes a fundamentally different approach to the Sanger chain-termination method that is the traditional method of sequencing. NGS leverages sequencing by synthesis (SBS) technology. SBS uses a DNA polymerase to catalyze the incorporation of fluorescently labeled deoxyribonucleotide triphosphates (dNTPs) into a DNA template strand during PCR. During each PCR cycle, the excitation of fluorophores can be used to identify which nucleotides were incorporated. NGS's works in a massively parallel fashion - instead of sequencing a single DNA fragment, millions of DNA fragments can be sequenced at once.

Surface plasmon resonance (SPR): This occurs as a consequence of the resonant, collective oscillation of conduction band electrons that are in resonance with the oscillating electric field of incident light. As a result, energetic plasmonic electrons are produced through non-radiative excitation.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

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