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Research Article

Milk MicroRNAs as a Biomarker of Bovine Mastitis

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Abstract

MicroRNAs (miRNAs) are non-coding small RNA molecules, which lengths are 19 - 24 nucleotides. MiRNAs can serve as biomarkers of many diseases. In this study, we aimed to identify the miRNAs in milk as biomarkers of bovine mastitis. The expression levels of milk miRNAs were analyzed using qRT-PCR and normalized to miR-92a. Six miRNAs (miR-21, miR-29b, miR-122, miR-146a, miR-155 and miR-383) were compared to normal and mastitis cow milk. MiR-21, miR-122, miR-146a, miR-155 and miR-383 were significantly up-regulated and miR-29b was significantly down-regulated in mastitis cow milk. In contrast, we found that miR-21, miR-122, miR-146a, miR-155 and miR-383 were significantly up-regulated in inflammation quarters (CMT+) than that of non-inflammation quarters (CMT-). This study demonstrates miRNAs as biomarker of bovine mastitis.

Keywords: *Mirnas; Bovine; Milk; Mastitis; Biomarker*

Introduction

MicroRNAs are about 22 nucleotides long non-coding small RNAs that play essential roles in gene expression through base-pairing with complementary sites at the 3'untranslated region in target mRNA molecules at the posttranscriptional level in plants and animals [1,2]. This binding inhibits translation or can cause degradation of target mRNA. The target seed sequence which is made of nucleotides 2 - 7 in miRNAs are deemed necessary for binding to target transcripts [3]. MicroRNAs can regulate multiple gene expression and in turn each mRNA can be regulated by several different miRNAs [4]. Most primary microRNAs are transcribed by RNA polymerase II and the primary miRNA hairpins are encoded in both protein coding and non-coding transcription units [5]. The mature microRNAs are guided to its target mRNA with partial complimentary binding sites on the 3' UTR causing repression or degradation of the mRNA [6].

Bovine mastitis is an infectious mammary gland disease that has been considered as one of the most economically important disease throughout the world for several decades [7]. Mastitis has significant economic impact in the global dairy industry, which is one of the most prevalent and costly diseases in dairy animals. Except direct losses which incurred by treatment and discarded milk during and after the treatment, sub-clinical mastitis decreases in milk yield and quality also cause indirect losses [8]. The reduction in milk production attributed to subclinical mastitis may responsible for approximately 70% of the total losses [9]. Discovering specific and sensitive biomarkers for mastitis early detection and treatment are with great social needs.

MiRNA regulate a post-transcriptional level of gene expression by causing RNA degradation or blocking translation of mRNA. MiRNAs play an important role in embryonic development, cell differentiation, immunity and senescence. MiRNAs are also correlated with dis-

eases such as cancer, viral diseases, immune-related diseases, liver and heart diseases. In one review introduce that miRNA have been investigated as noninvasive biomarkers of 20 different cancers, 11 organ damage conditions and 10 diverse disease states in human medicine [10]. Thus, miRNA have specific roles in disease pathogenesis.

MiRNAs have also been identified in milk of cow [11,12], pig [13], human [14], goat [15], rat [16], and yak [17]. MiRNAs are transferred from the mother's milk to the infant via the digestive tract and play a critical role in infant's immune system development [18,19]. MiRNAs in milk are resistant to acidic environments, RNase digestion, incubation at room temperature and various freeze/thaw cycles [12,18-21]. Chen X., *et al*. suggest that miRNAs can serve as a new standard for the quality control of milk-related commercial products [11].

MiRNA expression levels changing between healthy animals and patients are important research field of disease related miRNAs. Quantitative polymerase chain reaction (qRT-PCR) is a common used and indirect technology for miRNAs expression allowing only relative quantification, which measures Ct values and then normalize to internal control gene to calculate the relative expression of the target miRNAs.

In this study, we made an attempt to investigate miRNAs expression levels between normal cows, non-mastitis quarters of mastitis cows (CMT-), mastitis quarters (CMT+) of mastitis cows, mastitis cows (CMT+) before treatment, and mastitis cows (CMT-) after treatment using qRT-PCR to verify whether miRNAs could be the suitable biomarkers of bovine mastitis milk.

Materials and Methods

Preparation of milk (whey) sample

Bovine raw milk samples (5 - 10 mL) were collected in the field and then immediately examined using California Mastitis Test (CMT). The tested milk samples were frozen at -80°C and transported to the laboratory. The samples were thawed and centrifuged at 3000g for 15 minutes to remove the fat, cells and large debris. The defatted supernatant was then centrifuged at 15000g for 15 minutes to remove residual fat, casein and cell debris. Finally, the clear supernatant (whey) was recovered and stored at -80°C until use.

Extraction of total RNA

Total RNA was extracted from supernatant samples (300 µL) using mirVana PARIS Kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. Acid-Phenol: Chloroform (Ambion) extraction was performed two times to reduce protein contamination. Briefly, whey samples (300 µL) were mixed with equal volume of 2X denaturing solutions and incubated on ice for 5 minutes. In addition, Acid-Phenol: Chloroform was added to the homogenates following vortexing. Then the homogenates were centrifuged at 15000g for 5 minutes. After centrifugation the aqueous phase was recovered and again performed the Acid-Phenol: Chloroform isolation phase. The recovered aqueous phase was added with 1.25 volumes of 100% room temperature ethanol, mixed thoroughly, and then passed the lysate with ethanol mixture through RNA filter cartridge. Then the filter cartridge was washed according to the manufacturer's protocol, and RNA was eluted in 100 µL elution solution at 95°C. Isolated RNA was stored at -80°C for until use.

Quantification of miRNA by qRT-PCR

The complementary DNA (cDNA) was generated using TaqMan microRNA assays (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. qRT-PCR was performed using a TaqMan fast advanced master mix kit on a StepOne Plus (Applied Biosystems) with 10 µL final volume including 1 µL RNA-specific cDNA. The thermal cycling program was according to the manufacturer's protocol, and all the experiments were performed in duplicate. Expression levels were determined using the 2-△△CT method, and normalized to miR-92a. qRT-PCR reactions of undetermined Ct were assigned at Ct = 40.

Statistical analysis

Statistical significance between groups was analyzed using Student's t-test. All values were expressed as mean ± SD. The significance of differences was determined using One-way ANOVA and followed by Tukey's test. Tukey's test was used for multiple comparisons (GraphPad Prism 6.0 software). A P-values < 0.05 were considered statistically significant.

Results

MicroRNAs are endogenous, non-coding, 19 - 24 nucleotides long, double-standard small RNA molecules that play essential roles in gene expression. Eight normal cows (32 quarters) and 28 mastitis cows (94 quarters) were included in this study for analysis of miR-21, miR-29b, miR-122, miR-146a, miR-155 and miR-383 expression levels. The expression levels of these miRNAs were studied with qRT-PCR and normalized to miR-92a. It was observed that the relative expression levels of miR-21, miR-122, miR-146a, miR-155 and miR-383 were significantly up-regulated, whereas miR-29b was significantly down-regulated when compared between normal and mastitis cow. It was also observed that the relative expression levels of miR-21, miR-155 and miR-383 were highly expressed in mastitis cow milk (Figure 1).

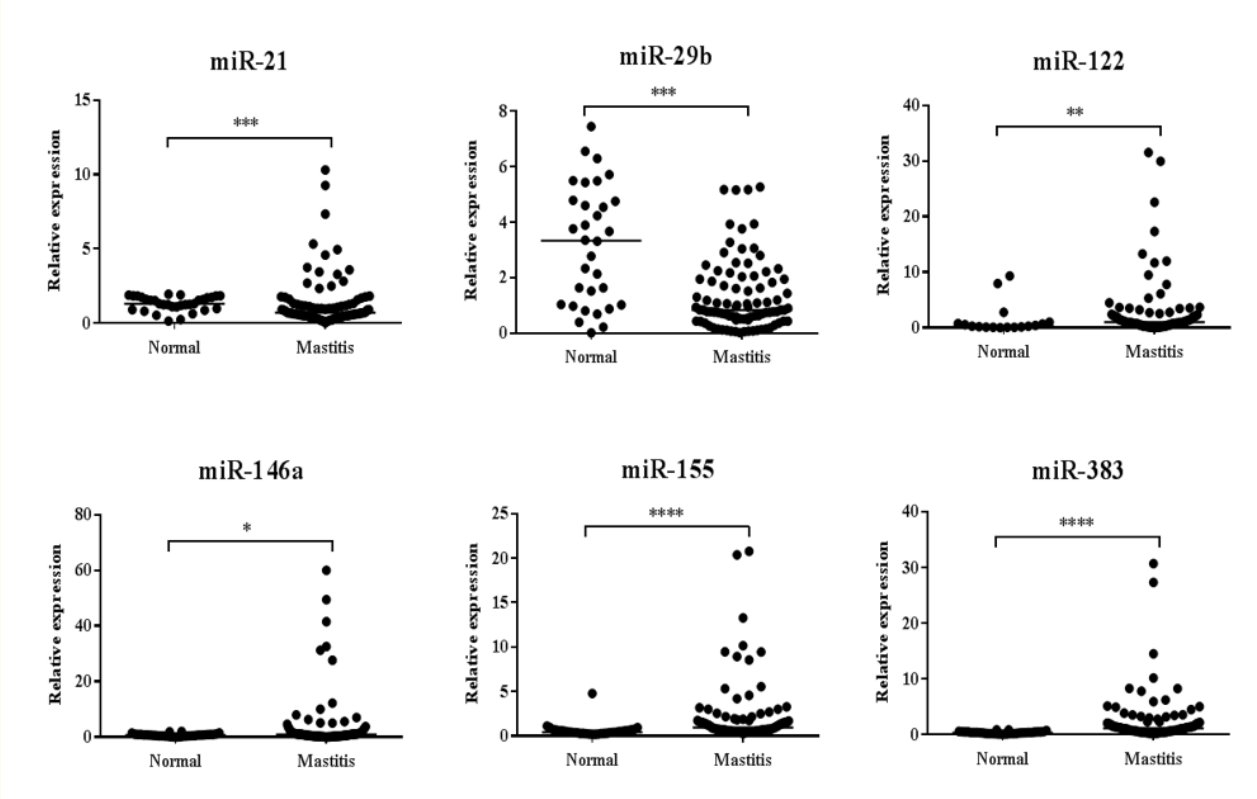


Figure 1: *Relative expression levels of miRNAs in bovine milk whey (supernatant) from normal cows, n = 32 from 8 cows; and mastitis cows, n = 94 from 28 cows. Horizontal bars indicate median values. The y-axis represents miRNA relative expression levels in arbitrary units. nsp > 0.05, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*

Thirty two quarters from eight normal cows; fifty eight quarters from eleven mastitis cows of non-mastitis quarters (CMT-) and thirty six quarters from seventeen mastitis cows of mastitis quarters (CMT+) were analyzed to identify the expression levels of miR-21, miR-29b, miR-122, miR-146a, miR-155 and miR-383 using qRT-PCR and normalized to miR-92a. MiR-122 and miR-383, which were highly upregulated in mastitis cows and inflammation related miR-21, miR-146a and miR-155, were also included as candidates. It was found that the relative expression levels of miR-21, miR-122, miR-146a, miR-155, and miR-383 were significantly up-regulated in CMT+ groups whereas miR-29b was significantly down-regulated when compared between normal and CMT+ groups. It was also found that the rela-

tive expression levels of miR-155 and miR-383 were highly expressed in CMT+ groups (Figure 2), however no significant difference was observed between before mastitis (B+) and after non-mastitis (A-) groups, except miR-122 (Figure 3).

Receiver operating characteristic (ROC) curve analysis using the relative expression levels of six miRNAs was performed to evaluate the ability in distinguishing normal from CMT+ groups (Figure 4). Area under the curve (AUC) analysis and Youden Index were applied to determine the optimal cut-off point, sensitivity and specificity of each miRNA. MiR-122, miR-155 and miR-383 had high predictive values (0.8 < AUC < 1); miR-21, miR-29b and miR-146a had moderate predictive values (0.5 < AUC < 0.8). MiR-21, miR-29b, miR-122, miR-146a, miR-155, and miR-383 generated a sensitivity of 0.3333, 0.7059, 0.9118, 0.6111, 0.7222 and 0.9143, and a specificity of 1, 0.7188, 0.7059, 0.8125, 0.9375 and 0.9063 in differentiating CMT+ milk from normal milk, respectively.

Discussion

Bovine mastitis is a worldwide problem and causes economical loss in dairy industry. California Mastitis Test (CMT), which is based on somatic cell count of milk, is a commonly use testing tool to detect mastitis. However, improving our understanding molecular pathogenesis of bovine mastitis would be beneficial to early diagnosis, treatment and prevention and that`s why it is needed to develop molecular biology based biomarker of bovine mastitis milk. There are few studies dealing with bovine mastitis related miRNAs. All of these studies

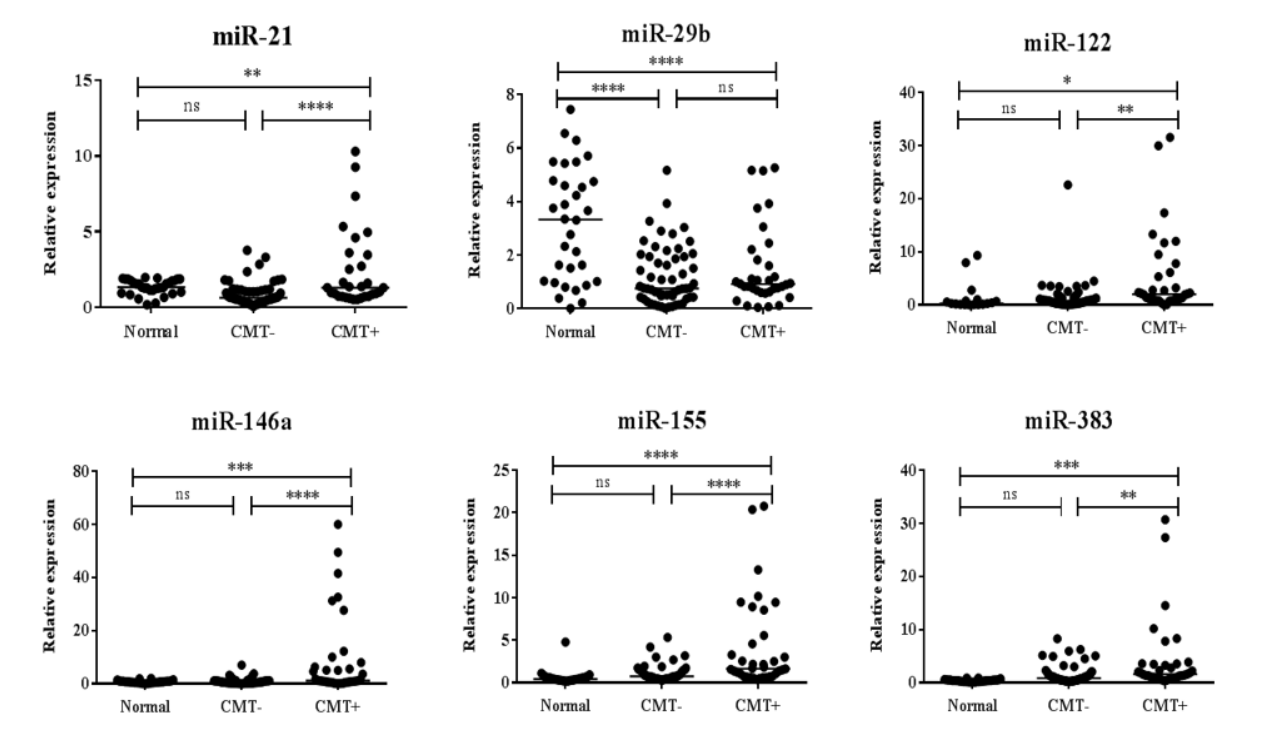
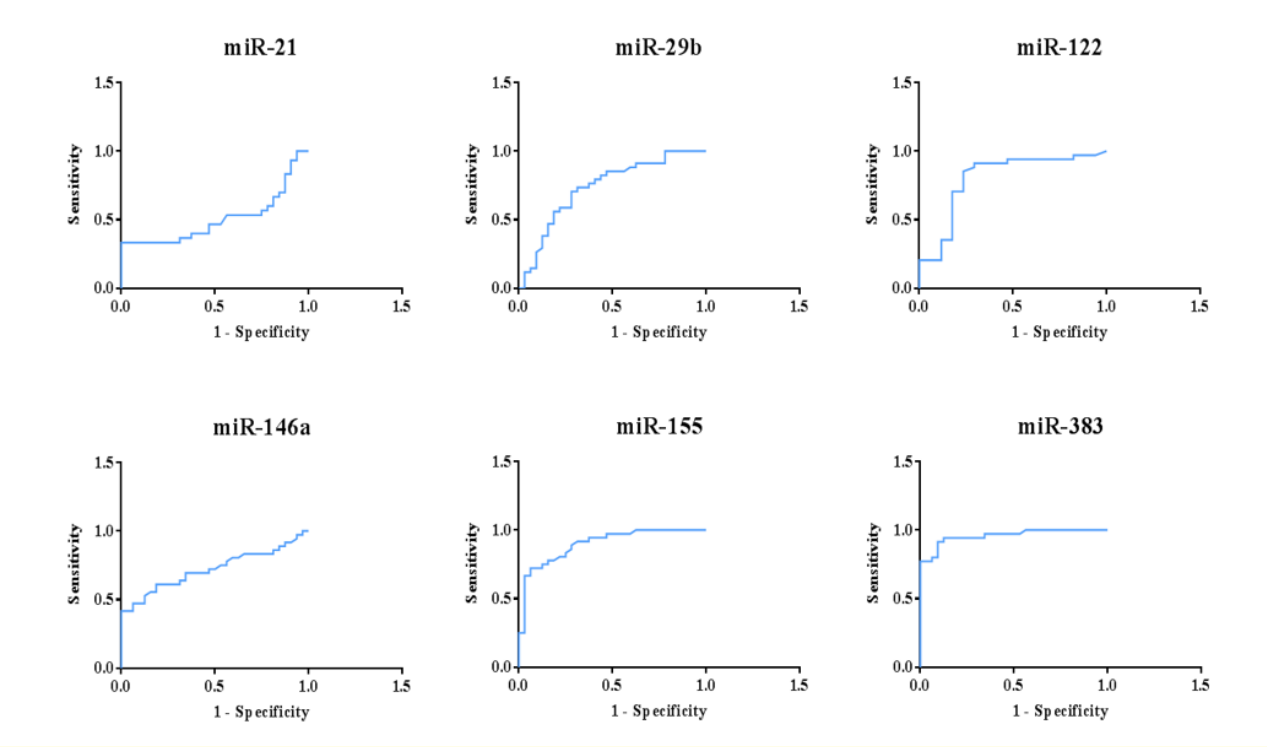


Figure 2: *Relative expression levels of miRNAs in bovine milk whey (supernatants) from normal cows, n = 32 from 8 cows; however, mastitis cows of non-mastitis quarters (CMT-), n = 58 from 11 cows; and mastitis quarters (CMT+), n = 36 from 17 cows. Horizontal bars indicate median values. The y-axis represents miRNA relative expression levels in arbitrary units. nsp > 0.05, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*



	miR-21	miR-29b	miR-122	miR-146a	miR-155	miR-383
AUC	0.5063	0.7362	0.8028	0.7218	0.8993	0.9585
Cut-off point	2.235	1.355	0.695	0.99	1	0.655
Sensitivity	0.3333	0.7059	0.9118	0.6111	0.7222	0.9143
Specificity	1	0.7188	0.7059	0.8125	0.9375	0.9063

Figure 3: *Bovine mastitis diagnostic values of miRNAs quantification by qPCR in milk. The ROC curve and AUC were used to analyze the relative expression levels of normal and CMT+ cow milk. Youden Index was applied to determine the optimal cut-off point, sensitivity and specificity.*

challenge the common mastitis pathogens *in vivo* or *in vitro* and observing miRNA change in bovine mammary epithelial cells [22-24] monocytes [25] or mammary gland tissue [26]. These studies examined the expression patterns of miRNAs within 48 hours after the pathogens challenged. However, clinical infections may persist for at least 2 months. Once chronic infection established, many of these infections persist for entire lactations or the life of the cow. The short-term pathogens challenge experiments (acute infection) may be unrepresentative of long-term infections (chronic infection). To the best of the authors' knowledge, this is the first report to compare milk miRNAs in health and mastitis cows.

To find the suitable miRNAs as biomarkers for mastitis milk, we used qRT-PCR technique to measure miRNAs expression in milk. Using qRT-PCR, we revealed that miR-21, miR-122, miR-146a, miR-155 and miR-383 were significantly up-regulated, whereas miR-29b

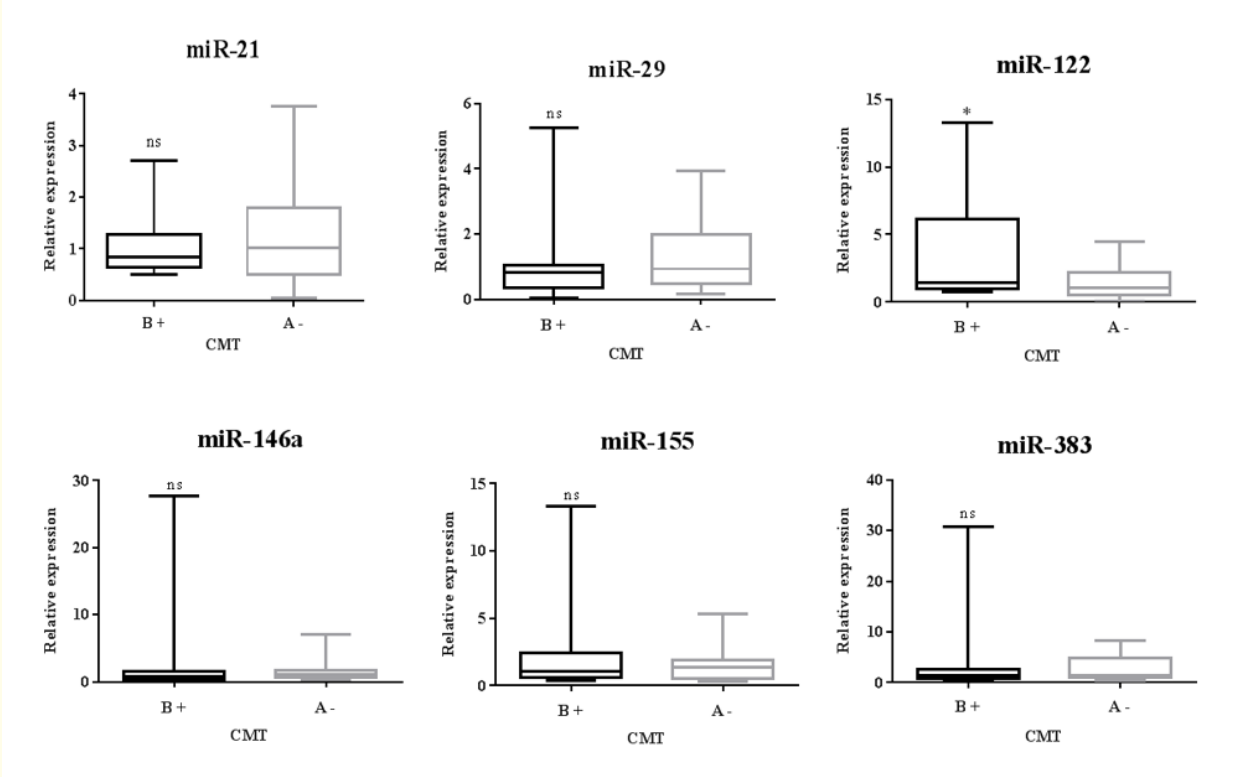


Figure 4: *Relative expression levels of miRNAs in bovine milk whey (supernatants) from mastitis quarters (CMT+) before treatment (B+), n = 17 from 17 cows; and mastitis quarters (CMT-) after treatment (A-), n = 17 from 17 cows. The values are expressed as mean ± SD. The y-axis represents miRNA relative expression levels in arbitrary units. nsp > 0.05, *p < 0.05.*

was significantly down-regulated in mastitis cow milk. We also revealed that miR-21, miR-122, miR-146a, miR-155 and miR-383 were significantly up-regulated in CMT+ milk.

In spite of digital PCR provides advantages of more accuracy and less ambiguity than qRT-PCR; however, qRT-PCR is less expensive and works over a much broader dynamic range [27,28]. The concentration of highly expressed miRNAs, such as miR-21 in this study, cannot be determined correctly using digital PCR without dilution. In addition to specificity and sensitivity, biomarkers should be accessible by noninvasive, cheap, and fast methods [29]. Thus, we analyzed levels of six miRNA biomarker candidates in milk using qRT-PCR. Receiver operating characteristic curve and area under the curve analysis suggested the diagnostic biomarker potential of five miRNA candidates (AUC values were between 0.5063 and 0.9585). All of the candidates generated good sensitivity and specificity in differentiating CMT+ milk from normal milk.

MiR-21, miR-146a and miR-155 are well known inflammation-related miRNAs. These miRNAs have been implicated in toll-like receptors 4 and nuclear factor kappa B (TLR4-NFκB) signaling pathway immune response. Of these inflammation-related miRNAs, miR-155 works to promotes immune response; however, miR-21 and miR-146a work to demote immune response [30]. MiR-383 dysregulation was reported to impact cell activity in tumor cells [31-34]; however, this is the first study observed that miR-383 was related with inflammation. The role of miR-383 involved in inflammatory response needs further studies to validate.

Conclusion

In conclusion, we demonstrated that dairy milk contains miRNAs. Besides, we showed that miRNAs expression patterns differ between normal cow milk and mastitis cow milk. In addition, we confirmed that miRNAs expression profile varies between mastitis cows in mastitis quarter (CMT+) and non-mastitis quarters (CMT-). Moreover, we showed that miRNAs expression levels differ between mastitis cows (CMT+) before treatment and mastitis cows (CMT-) after treatment. Here we confirmed that miRNAs expression profiles in dairy cows are regulated by veterinary disease like bovine mastitis and we can consider miR-21, miR-122, miR-146a, miR-155 and miR-383 as a biomarker of bovine mastitis. Additional studies are needed to confirm specific miRNAs as a biomarker for early detection bovine mastitis.

Declaration of Interest

The authors have no relevant interests to declare.

Bibliography

1. Ameres SL and PD Zamore. "Diversifying microRNA sequence and function". *Nature Reviews Molecular Cell Biology* 14 (2013): 475-488.
2. Lee Y., *et al*. "MicroRNA genes are transcribed by RNA polymerase II". *The EMBO Journal* 23.20 (2004): 4051-4060.
3. Lewis BP., *et al*. "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets". *Cell* 120.1 (2005): 15-20.
4. Iborra M., *et al*. "MicroRNAs in autoimmunity and inflammatory bowel disease: crucial regulators in immune response". *Autoimmunity Reviews* 11.5 (2012): 305-314.
5. Davis BN and A Hata. "Regulation of microRNA biogenesis: A miRiad of mechanisms". *Cell Communication and Signaling* 7 (2009): 18.
6. Yang JS and EC Lai. "Alternative miRNA biogenesis pathways and the interpretation of core miRNA pathway mutants". *Molecular Cell* 43.6 (2011): 892-903.
7. Hogeveen H., *et al*. "Economic aspects of mastitis: new developments". *The New Zealand Veterinary Journal* 59.1 (2011): 16-23.
8. Petrovski KR., *et al*. "A review of the factors affecting the costs of bovine mastitis". *Journal of the South African Veterinary Association* 77.2 (2006): 52-60.
9. Varshney JP and R Naresh. "Evaluation of a homeopathic complex in the clinical management of udder diseases of riverine buffaloes". *Homeopathy* 93.1 (2004): 17-20.
10. Saikumar J., *et al*. "Noninvasive Micromarkers". *Clinical Chemistry* 60.9 (2014): 1158-1173.
11. Chen X., *et al*. "Identification and characterization of microRNAs in raw milk during different periods of lactation, commercial fluid, and powdered milk products". *Cell Research* 20.10 (2010): 1128-1137.
12. Izumi H., *et al*. "Bovine milk contains microRNA and messenger RNA that are stable under degradative conditions". *Journal of Dairy Science* 95.9 (2012): 4831-4841.
13. Chen T., *et al*. "Exploration of microRNAs in porcine milk exosomes". *BMC Genomics* 15 (2014): 100.
14. Weber JA., *et al*. "The microRNA spectrum in 12 body fluids". *Clinical Chemistry* 56.11 (2010): 1733-1741.
15. Li Z., *et al*. "Expression profiles of microRNAs from lactating and non-lactating bovine mammary glands and identification of miRNA related to lactation". *BMC Genomics* 13 (2012): 731.

16. Izumi H., *et al*. "Time-dependent expression profiles of microRNAs and mRNAs in rat milk whey". *PLoS One* 9.2 (2014): e88843.

17. Bai WL., *et al*. "Technical note: identification of suitable normalizers for microRNA expression analysis in milk somatic cells of the yak (*Bos grunniens*)". *Journal of Dairy Science* 96.7 (2013): 4529-4534.

18. Kosaka N., *et al*. "MicroRNA as a new immune-regulatory agent in breast milk". *Silence* 1.1 (2010): 7.

19. Zhou Q., *et al*., "Immune-related microRNAs are abundant in breast milk exosomes". *International Journal of Biological Sciences* 8.1 (2012): 118-123.

20. Hata T., *et al*. "Isolation of bovine milk-derived microvesicles carrying mRNAs and microRNAs". *Biochemical and Biophysical Research Communications* 396.2 (2010): 528-533.

21. Gu Y., *et al*. "Lactation-related microRNA expression profiles of porcine breast milk exosomes". *PLoS One* 7.8 (2012): e43691.

22. Jin W., *et al*. "Transcriptome microRNA profiling of bovine mammary epithelial cells challenged with *Escherichia coli* or *Staphylococcus aureus* bacteria reveals pathogen directed microRNA expression profiles". *BMC Genomics* 15 (2014): 181.

23. Naeem A., *et al*. "Bioinformatics analysis of microRNA and putative target genes in bovine mammary tissue infected with *Streptococcus uberis*". *Journal of Dairy Science* 95.11 (2012): 6397-6408.

24. Lawless N., *et al*. "Next generation sequencing reveals the expression of a unique miRNA profile in response to a gram-positive bacterial infection". *PLoS One* 8.3 (2013): e57543.

25. Lawless N., *et al*. "MicroRNA regulation of bovine monocyte inflammatory and metabolic networks in an in vivo infection model". *G3 (Bethesda)* 4.6 (2014): 957-971.

26. Li R., *et al*. "Transcriptome microRNA profiling of bovine mammary glands infected with Staphylococcus aureus". *International Journal of Molecular Sciences* 16.3 (2015): 4997-5013.

27. Baker M. "Digital PCR hits its stride". *Nature Methods* 9.6 (2012): 541-544.

28. Hayden RT., *et al*. "Comparison of droplet digital PCR to real-time PCR for quantitative detection of cytomegalovirus". *Journal of Clinical Microbiology* 51.2 (2013): 540-546.

29. Dumache R., *et al*. "Use of miRNAs as Biomarkers in Sepsis". *Analytical Cellular Pathology* (2015): 186716.

30. O'Connell RM., *et al*. "microRNA regulation of inflammatory responses". *The Annual Review of Immunology* 30 (2012): 295-312.

31. Josson S., *et al*. "Radiation modulation of microRNA in prostate cancer cell lines". *Prostate* 68.15 (2008): 1599-606.

32. Boguslawska J., *et al*. "MiR-224 targets the 3'UTR of type 1 5'-iodothyronine deiodinase possibly contributing to tissue hypothyroidism in renal cancer". *PLoS One* 6.9 (2011): e24541.

33. Xu D., *et al*. "MicroRNA-383 expression regulates proliferation, migration, invasion, and apoptosis in human glioma cells". *Tumor Biology* (2015).

34. Zhao L., *et al*. "MicroRNA-383 regulates the apoptosis of tumor cells through targeting Gadd45g". *PLoS One* 9.11 (2014): e110472.

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