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J Vet Res 67, 583-591, 2023
DOI: 10.2478/jvetres-2023-0062

Droplet digital PCR quantification of selected microRNAs in raw mastitic cow's milk from the west of Poland

Sebastian Smulski[1]✉, Marcin Pszczoła[2], Monika Stachowiak[2], Adrianna Bilińska[2], Izabela Szczerbal[2]✉

[1]Department of Internal Diseases and Diagnostics, [2]Department of Genetics and Animal Breeding, Poznan University of Life Sciences, 60-637, Poznan, Poland
sebastian.smulski@up.poznan.pl
izabela.szczerbal@up.poznan.pl

Received: June 15, 2023 *Accepted: October 27, 2023*

Abstract

Introduction: MicroRNAs (miRNAs), a class of noncoding small RNAs, have been recognised as potential biomarkers of mammary gland conditions, including bovine mastitis diagnosis. The aim of this study was to quantify selected miRNAs in the milk of mastitic cows. **Material and Methods:** Milk samples (n = 90) were collected from healthy and mastitic dairy cows originating from local dairy cattle farms located in the west of Poland. MicroRNAs of the miR-21a, miR-92a, miR-146a and miR-383 species were quantified using the highly sensitive droplet digital PCR method. Direct measurement of somatic cell count (SCC) was performed using a cell counter. Cows were divided into three groups: those with an SCC below 200,000/mL were designated Low (n = 25), those with an SCC between 200,000 and 999,999 were Medium (n = 34), and those with an SCC of 1,000,000 or higher were High (n = 31). Microbiological analyses were performed using standard culture testing. **Results:** The level of miR-383 was very low and this miRNA was excluded from analysis. The miR-92a was used to normalise miR-21a and miR-146a expression levels. The obtained results of expression of miR-21a and miR-146a correlated with somatic cell number (R = 0.53 and 0.79, respectively). **Conclusion:** These results show that ddPCR is a useful method for quantifying miRNAs in raw cow milk. It seems that miR-146a is a promising marker for bovine mastitis, although further studies are needed to select a panel of miRNAs that can be used in mastitis monitoring in Poland.

Keywords: biomarker, bovine mastitis, ddPCR, microRNA, raw milk.

Introduction

Inflammation of the mammary gland, referred to as mastitis, is the most common disease in dairy cattle worldwide. It has been recognised as a major cause of economic losses in the dairy industry because it decreases milk production, impairs fertility, necessitates culling of affected animals, and imposes treatment cost (13, 30, 32). Mastitis is classified as clinical (CM) or subclinical (SCM), depending on the effects of the inflammation on the mammary gland. Although SCM has no visible effects on the udder or milk, it can affect milk composition and consequently product quality (28). Subclinical mastitis is approximately forty times more common than the clinical variety and is more difficult to detect (11). A well-known indicator of mastitis is somatic cell count (SCC) in milk, which reflects the health status of the udder (38). Usually, SCC values of over 200,000 cells/mL are considered to indicate SCM (12, 29). However, SCC in the milk can vary with many other factors, such as breed, age, parity, stage of lactation, body condition score, stress, and methods of transportation and storage of milk samples (31). A further demerit of SCC as a mastitis indicator is that the rise in the number of somatic cells appears after a significant delay in relation to the progress of inflammation (4). There are also other methods of determining the severity of inflammation (electrical conductivity of milk, lactose level and acute phase protein level); however, the results obtained by these methods are subject to significant error and so their usefulness is low. The search is in progress for alternative methods of assessing the severity of the

inflammation of the mammary gland which will allow the severity of mastitis to be identified with greater accuracy (7). Novel, sensitive, and accurate methods for the early detection of affected animals, especially those with mastitis in the subclinical stage, are therefore urgently needed (5).

MicroRNAs (miRNAs) are a class of small noncoding RNA molecules present in all body tissues and fluids; they have been recognised as useful biomarkers in the detection of many diseases (10, 33). In milk they can be present both as free molecules and packaged in vesicles, such as milk exosomes or fat globules (2). MicroRNAs encapsulated in vesicles or conjugated to RNA-binding lipoproteins are more stable and can be measured by a range of techniques, including quantitative PCR, digital PCR and high-throughput assays (25). A number of studies have identified miRNAs associated with mastitis in cattle (18, 19, 34, 37); however, different miRNAs have been proposed as mastitis biomarkers in different studies (24). The low repeatability of the results supporting certain miRNAs as viable biomarkers may be associated with the type of microorganism responsible for infection. The most common causes of mastitis are Gram-positive and Gram-negative bacteria, although viral, fungal and protothecal infections can also cause mastitis to develop (39). It has been shown that prevalence of individual pathogens isolated from milk samples can change over the years. For example, *Staphylococcus aureus* was the most commonly isolated bacterium in northeastern Poland in 2013, while from 2014 to 2019 *Streptococcus* spp. were the most common (15). Therefore, studies identifying miRNAs that correlate well with mastitis stage in groups in different locations are needed. To the best of our knowledge, studies using miRNAs from milk as predictors of mammary gland inflammation in cattle have not been conducted yet in Poland.

The aim of this study was to use the highly sensitive droplet digital PCR (ddPCR) method to quantify selected miRNAs (miR-21a, miR-92a, miR-146a and miR-383) in bovine milk with numbers of somatic cells characteristic of different microbiological profiles. These miRNAs were selected based on literature results that point to their frequent association with bovine mastitis.

Material and Methods

Animals and sample collection. Three commercial Holstein-Friesian dairy farms located in the western part of Poland were enrolled in the study. The management of all farms was similar, with all employing a free-stall system. The farms held 120, 400 and 850 milking cows which yielded on average 9,000 kg/305 days of lactation. A total of 50 dairy cows showing a subclinical form of mastitis and 50 cows with healthy udders were randomly selected for the experiment. One milk sample of approximately 10 ± 2 mL was collected from the diseased quarter of the udder of each enrolled cow with mastitis. Cows with mastitis in two or more quarters were excluded from the experiment in order to sample only one quarter. In the healthy cows, milk from the rear right quarter was collected. The milk samples were immediately screened in the field using a California mastitis test (CMT) with a commercial tester (Mastirapid, Vetoquinol Biowet, Gorzów Wielkopolski, Poland). Milk samples were cooled to 5–8°C and transported to the laboratory in approximately 30–50 min. Immediately upon delivery, the samples from the affected and healthy cows were analysed for somatic cell count using a DeLaval cell counter (DeLaval, Tumba, Sweden) and for microbiological quality. Identification of bacteria was performed in line with National Mastitis Council procedures (23). The milk samples were centrifuged at 3,000 × *g* for 15 min at room temperature to remove cell debris and fat prior to molecular analysis. The supernatant was recovered and centrifuged again at 15,000 × *g* for 15 min at 4°C. The milk whey was also recovered and stored at −80°C until RNA extraction.

Candidate microRNA selection. Four candidate miRNAs (miR-21a, miR-92a, miR-146a and miR-383) were selected on the basis of reports indicating that they are potential biomarkers of bovine mastitis. A preliminary study was performed for all miRNAs in a small group of affected animals (n = 3) and one of healthy animals (n = 3).

RNA extraction. Total RNA was extracted from 300 µL of milk whey using a miRNeasy Micro Kit (Qiagen, Hilden, Germany) and following the manufacturer's protocol. The RNA concentration was determined using a Qubit RNA HS Assay Kit on a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA).

MicroRNA-specific reverse transcription. Reverse transcription was performed with 10 ng of total RNA using a TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) and following the manufacturer's protocol. The following TaqMan MicroRNA Assays (Thermo Fisher Scientific) were employed: miR-21a-5p (assay ID 000397, catalogue no. 4427975), miR-92a-3p (assay ID 000431, catalogue no. 4427975), miR-146a (assay ID 005896, catalogue no. 4440886) and miR-383-5p (assay ID 000573, catalogue no. 4427975).

Droplet digital PCR (ddPCR). PCR reaction mixtures consisted of 2 µL of cDNA, 11 µL of 2 × ddPCR SuperMix for Probes (Bio-Rad Laboratories, Hercules, CA, USA), 9 µL of H_2O and 1 µL of TaqMan primers and probe from the corresponding TaqMan MicroRNA Assay. The reaction mixtures were divided into approximately 20,000 droplets using a QX200 droplet generator (Bio-Rad) and subjected to PCR in a T100 Thermal Cycler (Bio-Rad) under the following conditions: ramp rate of 2°C/s, initial denaturation

at 95°C for 10 min, 40 cycles at 94°C for 30 s and at 60°C for 1 min, and denaturation at 98°C for 10 min. A QX200 droplet reader (Bio-Rad) was used to detect fluorescence, and the results were analysed using QuantaSoft software (Bio-Rad). The fraction of positive droplets was quantified using the Poisson distribution.

Statistical analysis. To determine the relationship between the miRNA concentration and relative expression levels, classes were created based on the SCC score in two scenarios. In the first scenario, the following three classes we used: Low (SCC < 200,000/mL), Medium (SCC ≥ 200,000/mL and SCC < 1,000,000/mL) and High (SCC ≥ 1,000,000/mL). In the second scenario only two classes were used: Low (SCC < 200,000/mL) and High (SCC ≥ 200,000/mL). The relationship was tested using linear regression analysis on ungrouped data after the dataset had been divided into the classes. Before performing the regression analysis, the expression, concentration and SCC levels were log-transformed to give a normal distribution. Regression analyses were performed using R software (27).

To analyse the differences between the miRNA expression levels and the presence of pathogens, the data were divided into three groups (Control, Positive and Negative) using the combined information on the SCC and the presence of bacteria in the samples. The Control group (n = 17) consisted of samples in which the SCC was low and no bacterial growth was observed. The Negative group (n = 33) consisted of samples in which the SCC was medium (n = 15) or high (n = 18) and bacterial growth was not observed. The Positive group (n = 15) consisted of samples in which the SCC was medium (n = 5) or high (n = 10) and bacterial growth was observed. The presence of differences was assessed by analysis of variance with R software (27). Whenever the analysis of variance yielded significant results, it was followed by pairwise comparisons using a *t*-test with pooled SD, as implemented in R. The P-values were adjusted for multiple testing using the Benjamini–Hochberg correction (3). The level of expression was log-transformed to normalise the distribution before the analysis.

A separate analysis was performed to investigate if the expression of miR-146a is suitable for predicting SCC class. This analysis applied one of the most popular non-parametric classification algorithms, *i.e.* the k-nearest-neighbour (knn) algorithm available in the caret package in R (16). This algorithm uses neighbour points to predict target class. The expression data were centred and scaled prior to analysis. The whole dataset was partitioned into a training subset (70% of the data) and a testing subset (70% of the data) at random. Training was performed using ten-fold cross-validation repeated three times, selecting the optimal number of neighbours. The analysis was performed for two scenarios with different grouping into SCC classes (Low, Medium and High in scenario 1 and Low and High in scenario 2). The performance of the knn algorithm was assessed using sensitivity (the proportion of observations correctly assigned a positive result out of all observations having this result for a given class), specificity (the proportion of observations correctly assigned a negative result out of all observations having this result for a given class), positive predictive value (the proportion of observations with a positive test result that truly do have the outcome of interest), negative predictive value (the proportion of observations with a negative test result that truly do not have the outcome of interest), and balanced accuracy (*i.e.* the arithmetic mean of sensitivity and specificity).

Results

Milk quarter samples were collected from 100 cows, but 10 cows with clinical mastitis were excluded because clots in the raw milk made it impossible to obtain an SCC. Cows were described in terms of their SCC and CMT parameters. During CMT testing, 38 cows were determined to be healthy and 52 cows' mastitis was confirmed. After verification by SCC, a difference between the mastitic status indicated by the CMT and the actual inflammation state (Low, Medium or High) based on the SCC was noted. There was only 1 positive CMT test among the Low cases, but there were 14 negative CMT tests among the Medium and High cases.

Bacteriology of milk samples. In the samples collected from the healthy udders (n = 25), 18 samples showed no growth and 7 showed contamination. The prevalence of pathogens isolated from the mastitic milk (n = 65) is presented in Table 1.

Table 1. Prevalence of pathogens isolated from mastitic-quarter milk samples

Pathogen	n	Prevalence (%)
Streptococcus uberis	6	9.23
Streptococcus dysgalactiae	1	1.54
Staphylococcus aureus	1	1.54
Coagulase-negative staphylococci	4	6.15
Escherichia coli	1	1.54
Klebsiella spp.	2	3.08
No growth	33	50.77
Contamination	17	26.15

A lack of bacterial growth was relatively frequent. The most common pathogens were *Streptococcus uberis* (9.23%) and coagulase-negative staphylococci (6.15%). A high proportion of contamination was observed.

Analysis of miRNA in milk samples. The expression of all miRNAs was successfully detected using ddPCR, but the level of miR-383 was very low; therefore, this miRNA was excluded from further analysis.

Since ddPCR allows absolute quantification of miRNAs, in the first step, the miR-21a, miR-92a and miR-146a concentrations were examined. It has been shown that all these molecules are upregulated in mastitic milk. In the next step, the level of miRNA concentration (directly measured) was analysed in relation to the SCCs. In the case of miR-21a, the correlation between concentration and SSC was quite low in all analysed samples (R = 0.32, P-value < 0.01) (Fig. 1), and was probably driven by two outliers from the High class.

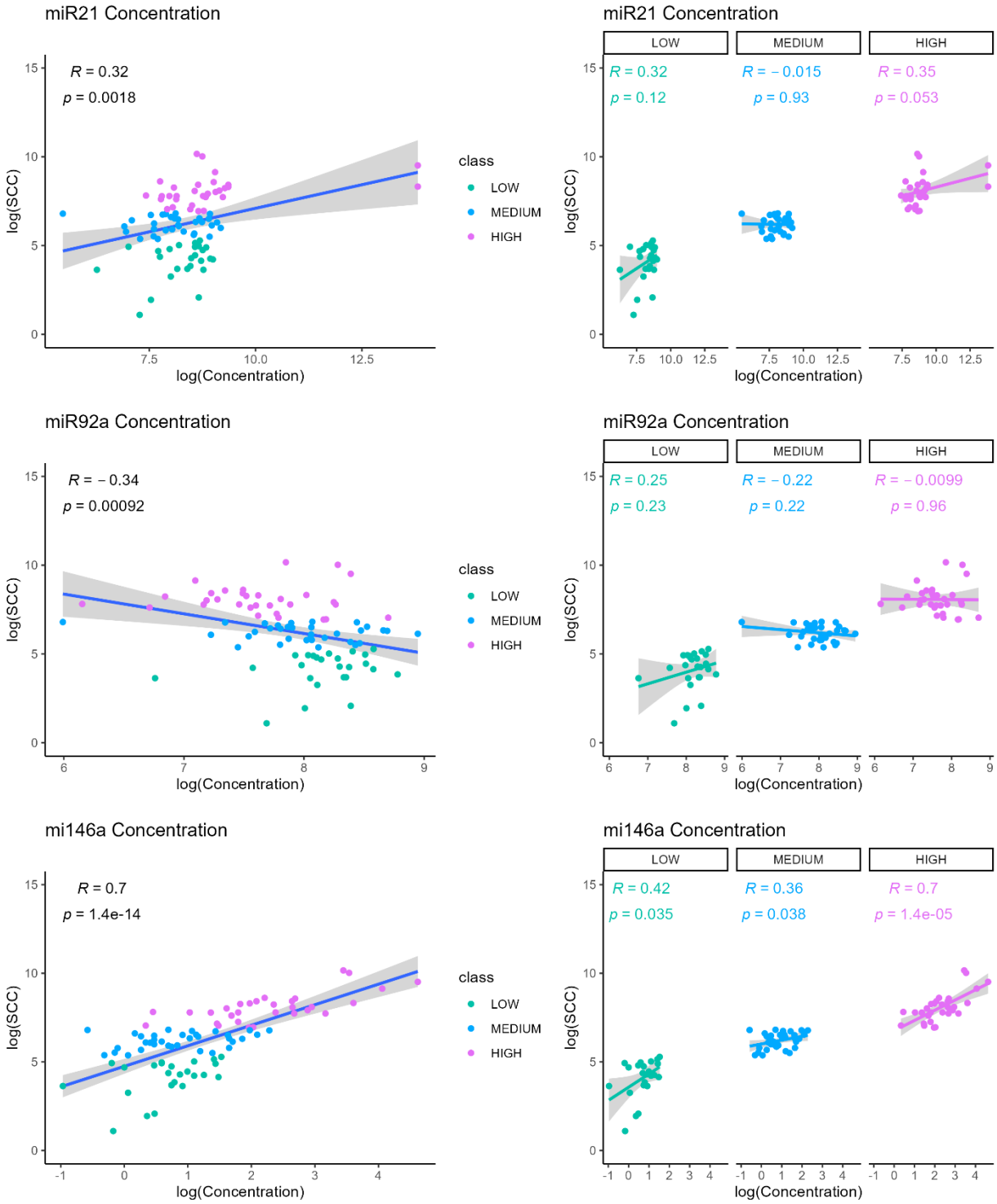

Fig. 1. Correlation between log(SCC) and concentrations of miR-21a, miR-92a and miR-146a in healthy and mastitic milk samples. The grey area below the figures indicates the 95% confidence interval. Low – somatic cell count < 200,000/mL; Medium – 200,000/mL ≤ somatic cell count < 1,000,000/mL; High – somatic cell count ≥ 1,000,000/mL

The correlation was not found to be statistically significant when the milk samples were divided into the three Low, Medium and High SCC groups (Fig. 1). In the case of miR-92a, a negative correlation (R = −0.34, P-value < 0.01) was observed when all the samples were analysed together (Fig. 1), and no significant results were found when the samples were divided into the three groups (Fig. 1). The most promising results were obtained for the concentration of miR-146a, where the correlation with SSC was high (R = 0.71, P-value < 0.01) for all samples (Fig. 1). When these were divided into the three SSC groups, a statistically significant correlation was observed for the Low (R = 0.44, P-value < 0.05) and High groups (R = 0.7, P-value < 0.01) (Fig. 1).

Although ddPCR allows absolute quantification of nucleic acids, various normalisation methods are recommended to avoid technical errors. In acknowledgement of this, it was decided to show results not only as concentrations but also as relative expression levels. One of the examined miRNAs, miR-92a, has been proposed as a housekeeping gene in the analysis of bovine mastitis–related microRNA in milk (17); it was used to normalise miR-21a and miR-146a expression (Fig. 2).

The correlation between SCC and miRNA relative expression level for the samples analysed together was as follows: R = 0.53 (P-value < 0.01) for miR-21a and R = 0.79 (P-value < 0.01) for miR-146a (Fig. 2). When the samples were divided into the three SSC groups, a correlation was observed for miR-21a in the High group (R = 0.44; P-value < 0.05) (Fig. 2) and for miR-146a in the Medium (R = 0.59, P-value < 0.01) and High groups (R = 0.7, P-value < 0.01).

Taking the correlations all together, miR-146a proved to be the most promising potential biomarker for the diagnosis of mastitis in the samples, since a positive correlation was observed between concentration and relative expression of this miRNA and SSC milk samples for all the samples, including when the samples were grouped. The strongest correlation was observed in the High group.

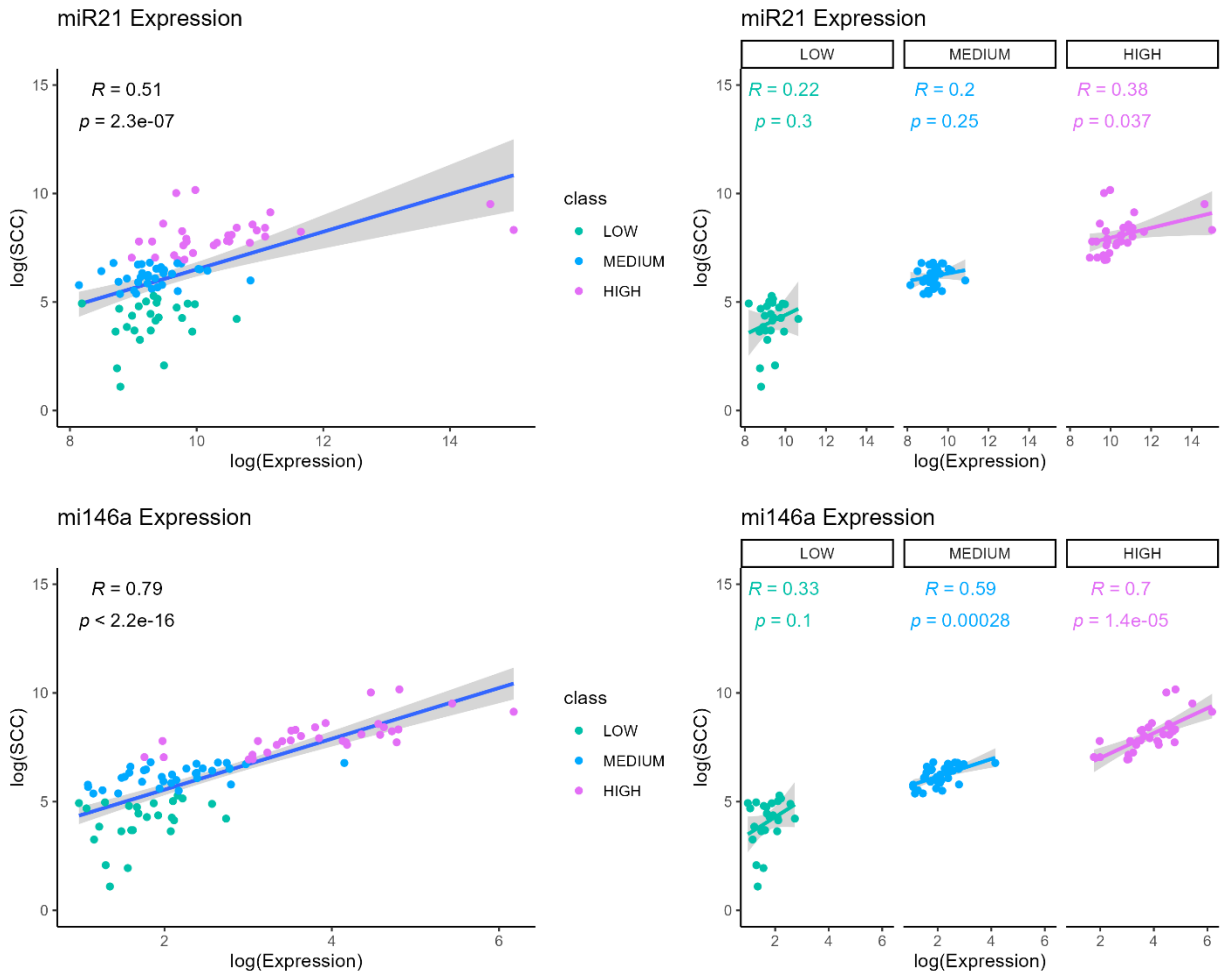

Fig. 2. Correlation between log(SCC) and the relative expression levels of miR-21a and miR-146a in healthy and mastitic milk samples. The grey area below the figures indicates the 95% confidence interval. Low – somatic cell count < 200,000/mL; Medium – 200,000/mL ≤ somatic cell count < 1,000,000/mL; High – somatic cell count ≥ 1,000,000/mL

Correlation between the isolated mastitis pathogens and the examined miRNAs. Comparison of the miR-21a relative expression levels across the Control, Negative and Positive groups showed no significant differences (P-value = 0.08). For miR-146a, the relative expression levels between the groups differed significantly (P-value < 0.01; Fig. 3). Multiple comparisons revealed that the expression level did not differ between the Positive and Negative groups (P-value = 0.9), but that the level in the Control group was significantly different to those of the other two (P-value < 0.01).

Predicting SCC class from miR-146a expression. The knn algorithm was employed to assess the possibility of predicting the SSC class from the relative expression of the miR-146a gene. A prediction was made for both scenarios (with two and three classes). The analysis performed on two classes provided a more accurate prediction of health status, with an accuracy of 0.97 and a 95% CI of (0.89, 0.99); when three classes were used, the accuracy equalled 0.72 and the 95% CI (0.59, 0.82). Other performance indicators were also superior for the two-class scenario (Table 2). In the three-class scenario, the accuracy of predicting the High class was 0.9.

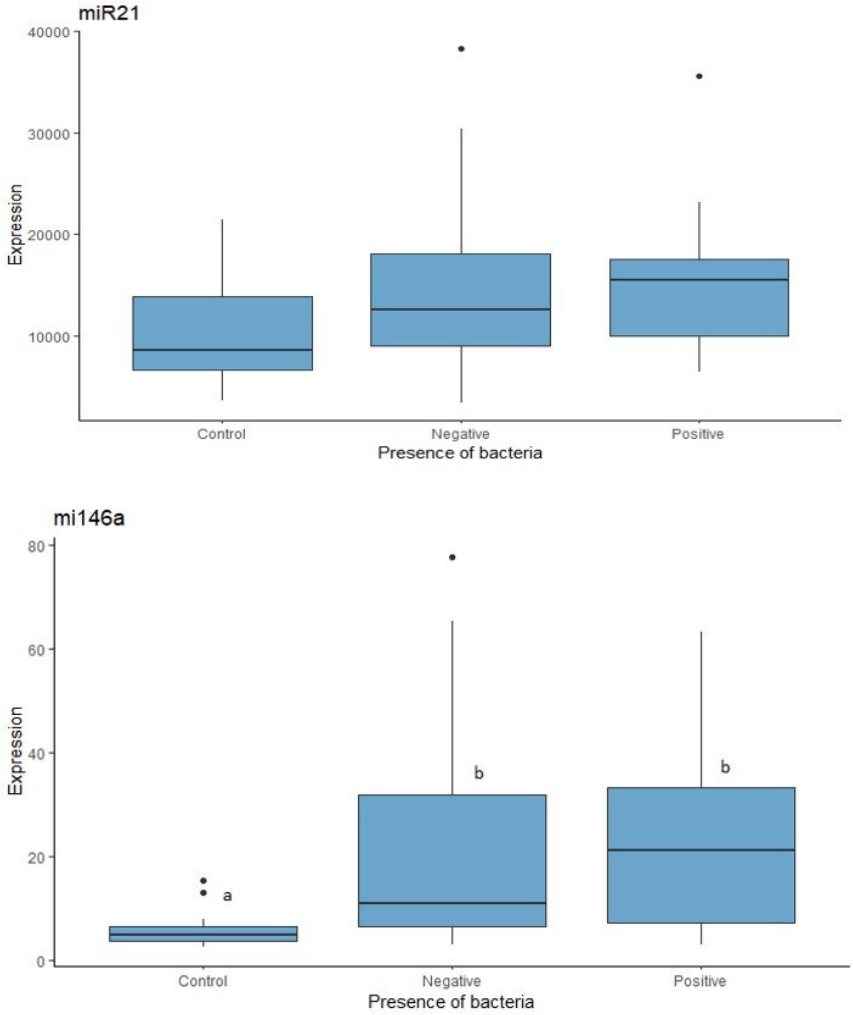

Fig. 3. Relative expression levels of miR-21a and miR-146a in three groups based on somatic cell counts and presence of a mastitoge. a, b – significant differences (P-value < 0.05)

Table 2. Performance of the K-nearest neighbour regression model, by somatic cell count class in healthy and mastitic milk samples

Parameter	Three-class analysis			Two-class analysis
	Low	Medium	High	Low
Sensitivity	0.67	0.67	0.82	0.98
Specificity	0.85	0.75	0.98	0.95
Positive predictive value	0.63	0.62	0.95	0.98
Negative predictive value	0.87	0.79	0.91	0.95
Balanced accuracy	0.76	0.71	0.90	0.97

Low – somatic cell count < 200,000/mL; Medium – 200,000/mL ≤ somatic cell count < 1,000,000/mL; High – somatic cell count ≥ 1,000,000/mL

Discussion

The CMT is currently the most common method of assessing the severity of bovine mastitis. It suffers from low sensitivity (<50%) but has the advantage that it can be performed under field conditions (9). Another common method of assessing the severity of inflammation of the mammary gland is through determination of the SCC of raw milk. This method is much more precise, but requires laboratory conditions. It also has the disadvantage that the increase in SCC follows the development of inflammation with a significant delay (1). Alternative methods of diagnosing mastitis in dairy cattle are thus needed. MicroRNA has recently been recognised as a potential diagnostic tool in the detection of bovine mastitis (24). Although many studies of miRNA expression levels in bovine milk have been carried out, most of them have evaluated mastitis status using CMTs, whereas our study employed both CMTs and SCC. In our experiment, 14 negative CMT results proved false, as SCC showed mastitis in these animals (SCC ≥ 200,000 cells/mL). Comparing the expression level of miRNA to the CMT results may thus introduce a significant error. We therefore decided to use only SCC data in the final analysis. We examined the potential role of miRNA for detecting mastitis on Polish cattle farms, and to the best of our knowledge investigated this as the first to do so. We also examined the milk samples bacteriologically, a step which may be highly productive when there is a relationship between a specific pathogen and a specific miRNA's expression. However, in this case, bacteriological examination did not allow us to find a significant difference between bacterial and nonbacterial mastitis in the expression level of miR-21a or miR-146a.

The results of the study by Lai *et al.* (18) and of other studies led us to initially select four miRNA molecules (miR-21a, miR-92a, miR-146a and miR-383) for the research. It has been shown that miR-21 expression in blood is upregulated in cows with mastitis caused by *Staphylococcus aureus* (6). Moyes *et al.* (21) suggested that the inflammatory response in the mammary gland involves crosstalk with the liver to coordinate the inflammatory response. Sensory neuron–derived exosomes containing miR-21a can increase the expression of proinflammatory genes and proteins when phagocytosed by macrophages. This result suggests that miR-21a plays an important role in communication between systemic organs in fighting infections of the mammary gland. Our results showed low levels of correlation between miR-21a and SCC. Similar conclusions were made by Naeem *et al.* (22), who found no significant difference in miR-21a expression levels between cows with mastitis caused by *Streptococcus uberis* and those with a healthy udder. On the other hand, Li *et al.* (20) found that mastitis caused by *Staphylococcus aureus* resulted in a 1.7-fold increase in miRNA-21a expression levels.

The ribonucleic acid miR-92a has been recognised as pro-inflammatory miRNA that is involved in the upregulation of several proinflammatory genes in macrophages (8). Our study did not find a correlation between the concentration of this molecule and SCC. Thus, following the recommendation of Lai *et al.* (17), the level of miR-92a was treated as a housekeeping gene level for the analysis of bovine mastitis-related microRNA in milk.

Our preliminary studies did not confirm that miR-383 is upregulated in mastitic milk; therefore, this molecule was excluded from the experiment. However, other studies found that miR-383 was significantly upregulated in CMT-positive milk (18) and in mammary epithelial cells in *Streptococcus agalactiae*-induced mastitis (26).

Many studies have examined the use of miRNA-146a in diagnosing bovine mastitis in milk and serum samples. As mentioned, Lai *et al.* (18) indicated that there were changes in the expression levels of this miRNA in mastitic cows confirmed by CMT. However, the number of animals in that experiment (18 CMT− animals and 17 CMT+ animals) was quite small. The same research group performed a genome-wide miRNA analysis of normal milk samples and milk samples from cows with clinical mastitis, also confirmed by CMT. They found 25 differentially expressed miRNAs during bovine mastitis, only three of which (miR-21a, miR-146a and miR-222) were the same as in the report published previously (19). In another study, that of Srikok *et al.* (34), eight *a priori* selected miRNAs were evaluated; three of them – miR-29b-2, miR-146a and miR-155 – were found to be good candidates to determine the infected status of cows using milk samples. Promise has been attributed to miR29b-2 as a biomarker for bovine mastitis. The recent study of Tzelos *et al.* (37) examined four miRNAs (miR-26a, miR-142, miR-146a and miR-223) in a large number of individual quarter milk samples (n = 236) collected from dairy cows at different lactations. They found that levels of miR-142, miR-146a and miR-223 could be used to identify the early stages of inflammation in individual quarter milk samples with high accuracy.

The examples presented above indicate that miR-146a may indeed be a good biomarker of bovine mastitis. It is known that this molecule regulates various biological pathways, including immunity, the development and function of immune components (14), and the response to microbial infection (35). For example, the expression level of miR-146a in B and T lymphocytes is an induced response to microbial infection (36). In our study, miR-146a showed differential expression between the control and experimental groups. High sensitivity (0.98) and specificity (0.95) were noted only when the cut-off value between mastitic and non-mastitic milk samples was an SSC of 1,000,000/mL or higher. The ability to correctly predict positive and negative results was also

high and was reflected in sensitivity of 0.95 and specificity of 0.98. Our results allow us to conclude that the level of expression of miR-146a is a good indicator of the severity of inflammation of the udder, especially for more severe inflammations, where the SCC in the tested milk is 1,000,000/mL or above. Using miR-146a to detect the severity of inflammations where the SCC is between 200,000 and 999,999/mL is much less sensitive (0.67) and specific (0.85). Our results indicate a need for further studies to identify new inflammatory markers for detecting inflammation in cases where the SCC is greater than 200,000/mL. In this study, we performed the first analysis of selected miRNAs in milk samples from healthy Polish cows and Polish cows suffering from clinical or subclinical mastitis confirmed using SCC. We showed that ddPCR is a useful method of quantifying the tested miRNAs in milk samples. As markers for bovine mastitis, miRNAs and miR-146a proved promising, but it is necessary to seek new markers that would allow identification of subclinical bovine mastitis.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: This study was financed by the National Science Centre, Poland under the Miniatura 2020/04/X/NZ9/00742 grant, and by the statutory funding of the Department of Genetics and Animal Breeding, Poznań University of Life Sciences, Poland (no. 506.534.09.00).

Animal Rights Statement: None required.

Acknowledgements: We thank Kacper Libera DVM, Magdalena Kubiak M.Sc., Joanna Stachecka PhD, and Magdalena Noak M.Sc. for their help with the sample collection and laboratory analysis.

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