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Expression Profiles of Inflammation-related MicroRNAs in *Mycoplasma bovis* Infected Milk of Holstein-Friesian and Doğu Anadolu Kırmızısı Cows

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ABSTRACT

Mycoplasma bovis is an important pathogen associated with several clinical diseases in cattle, such as mastitis, arthritis, and pneumonia. TableIn this study, we aimed to identify miRNA candidate biomarkers associated with inflammation in *Mycoplasma bovis* -infected milk samples and normal milk samples of Holstein-Friesian (HF) and Doğu Anadolu Kırmızısı (DAK) cows in Turkey. The expression levels of miRNAs in milk from mastitis-infected cows and uninfected cows were analyzed using a qRT-PCR. The results revealed that miR-21, miR-146a, miR-155, miR-222, miR-383, miR-200a, miR-205, miR-122, and miR-182 were upregulated in mastitis milk. Among the miRNA candidate biomarkers, miR-21 and miR-222 were significantly upregulated only in mastitis milk samples from HF cows, and miR-146a and miR-383 were significantly upregulated only in mastitis milk samples from DAK cows. These results shed light on miRNA candidate biomarkers in milk from HF and DAK cows with subclinical mastitis. The upregulated miRNAs detected in the present study could be used as biomarkers in the diagnosis of subclinical mastitis caused by *Mycoplasma bovis*.

Research Article

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Siyah Alaca ve Doğu Anadolu Kırmızısı Irkına Ait Sığırların *Mycoplasma bovis* ile Enfekte Sütlerinden Köken Alan Eksozomlardaki Yangı Ile Ilişkili miRNA'ların Ekpresyon Profili

ÖZET

Mikoplazma bovis, sığırlarda mastitis, artrit ve pnömoni gibi çeşitli klinik hastalıklarla ilişkili önemli bir ajandır. Bu çalışmada, Türkiye'de yetiştirilen Siyah Alaca (SA) ve Doğu Anadolu Kırmızısı (DAK) sığırlarına ait Mycoplasma bovis ile enfekte subklinik mastitli ve normal sütlerde inflamasyon ile ilişkili miRNA adaylarının belirlenmesi amaçlandı. Mastitli ve normal sığırlardan elde edilen sütteki miRNA'ların ekspresyon seviyeleri qRT-PCR ile analiz edildi. MiR-21, miR-146a, miR-155, miR-222, miR-383, miR-200a, miR-205, miR-122, miR-182'nin ekspresyon düzeylerinin her iki sığıra ait mastitli sütte arttığı gözlendi. Bununla birlikte, miR-21 ve miR-222'nin Holştayn sığırının mastitli sütünde önemli ölçüde arttığı, miR-146a ve miR-383'ün ise DAK sığırının mastitli sütünde önemli ölçüde arttığı belirlendi. Sonuç olarak, subklinik mastitli sütte ekspresyon düzeyi artan miRNA adayları Holştayn ve DAK sığırlarında belirlendi. Araştırmadan elde edilen bulgular, subklinik mastitis sütünde ekspresyon düzeyi artan miRNA'ların *Mycoplasma bovis*'in neden olduğu subklinik masitit tanısında biyobelirteç olarak kullanılabileceğini göstermiştir.

Araştırma Makalesi

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Anahtar Kelimeler
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INTRODUCTION

Mastitis is defined as an inflammatory response caused by infection of mammary gland tissue. Mastitis occurs in many mammalian species, especially in dairy cows (Gomes and Henriques, 2016). Mastitis in dairy cows causes direct and indirect economic losses. Direct losses are caused by treatment costs, un-used milk,

personnel expenses, deaths, and recurrence of mastitis. Indirect losses are due to decreased milk yields and milk quality, increased separation processes, decreased animal welfare, and other health problems (Petrovski et al., 2006). Infections with *Enterobacter spp.* including s*taphylococci* and *streptococci*, are responsible for the majority of mastitis cases. Recent studies reported that *Mycoplasma bovis* (*M. bovis*) caused mastitis in dairy cows (Rossetti et al., 2010; Wisselink et al., 2019; Appelt et al., 2019; Behera et al., 2018; Cai et al., 2005; Vahanikkila et al., 2019; Murai and Higuchi, 2019; Al-Farha et al., 2018; Al-Farha et al., 2017).

Mastitis can be divided into clinical and subclinical. The risk of contamination in cases of clinical mastitis can be averted by separating the infected animal from the herd. However, in subclinical mastitis, the animal does not show clinical signs at an early stage and therefore remains in the herd, allowing mastitis agents to be transmitted to other animals in the herd (Gussmann et al., 2019a; Gussmann et al., 2019b). The latter can result in severe economic losses (Jiang et al., 2019). A number of biomarkers, including NAGase, serum amyloid A, haptoglobin, and cytoplasmic enzymes (e.g., lactate dehydrogenase), can be used to detect subclinical mastitis. (Issaq and Blonder, 2009). Recently, biomarker efforts have been thought for use as biomarkers for the detection of microRNAs (miRNAs) found in milk microvesicles of cows with mastitis. Identification of miRNA markers that can be used in the diagnosis of mastitis would offer an effective alternative (Deb et al., 2013).

MiRNAs are small RNAs, about 22 nucleotides in length, that regulate gene expression by binding to the complementary sequence of the target mRNA or the 3'UTR region. They originate from precursor miRNAs composed of 70 nucleotides (Berezikov 2011; Jin et al., 2014). Recent studies reported that some miRNAs associated with inflammation (i.e., miR-21, miR-146a, miR-155, miR-222, miR-383, miR-200a, miR-205, miR-122, and miR-182) were highly expressed in mastitis milk (Lai et al., 2017; Luoreng et al., 2018). The aim of this study was to determine the expression levels of these miRNAs in milk from Holstein-Friesian (HF) and Doğu Anadolu Kırmızısı (DAK) cows with subclinical mastitis caused by *M. bovis* and uninfected milk from these animals.

MATERIALS and METHODS

Sample collection

Milk samples were collected from HF (n = 40; Healthy=6, Infected=34) and DAK (n = 40; Healthy=7, Infected=33) cows in third lactation. Milk from one cow was treated as one specimen. The California Mastitis Test (CMT) (Bergonier et al., 2003) and milk somatic cell counts by Coulter (Miller et al., 1986) were performed to detect mastitis cases. Addition, it was evaluated whether clinical symptoms were present or not. CMT ++ and CMT +++ cows with somatic cell counts of more than 200,000 in at least one-quarter and cows with no clinic symptoms served as the subclinical mastitis group. CMT+ cows with a somatic cell count of less than 200,000 in all quarters (cranial and caudal and left and right side) served as the normal group (Tables 1 and 2). After collection, the milk samples were transferred into sterile falcon tubes. The milk samples were stored at -80° C until the analysis.

Detection of mastitis pathogens using the RT-PCR method

Four mastitis-causing pathogens, *Staphylococcus aureus*, *Streptococcus agalactiae*, *M. bovis*, and other Mycoplasma spp., were detected in milk samples using a VetMAX™ MastiType Myco8 Kit (Thermo) according to the manufacturer's protocol. DNA isolation from all the milk samples was performed using a MagMAX DNA Multi-Sample Ultra Kit 2.0 (Thermo) according to the manufacturer's protocol. MastiType Positive Control was used as a positive control, and nuclease-free water instead of sample DNA was used as a mastitis negative control. The RT-PCR conditions were 95° C for 10 min, 95° C for 5 s, and 60° C for 1 min for 40 cycles.

Verification of M. bovis using the RT-PCR method

The primer sequences of the *uvrC* gene of *M. bovis* were obtained from a previous study: Mbov_uvrC_R, 5′-GAATTTACGCAAGAGAATGCTTCA-3′; Mbov_uvrC_R, 5′-GCAATGCCTCTTTATTTGTTTTACAG-3′ (Rossetti et al. 2010). To detect the *uvrC* gene in *M. bovis*-positive milk samples, an RT-PCR assay was performed using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). The reaction volume was 25 µl. The mixture was as follows; 12.5 µl of QuantiTect SYBR Green (Qiagen, Germany), 1 µl of forward and reverse primers (100 nmol), 2 µl of milk lysates, and 8.5 µl of ultrapure water. The PCR conditions were as follows: 95° C for 10 min, 95° C for 15 s, 60° C for 30 s, and 72° C for 30 for 40 cycles. The PCR products were analyzed in 1% agarose gel.

Exosome isolation

First, 5 ml of *M. bovis* negative and *M. bovis*-positive milk were centrifuged at 2,500 × g for 10 min to remove cells and fat deposits. Subsequently, the supernatant was centrifuged at 12,000 ×g at 4° C for 30 min to remove cellular residues. The supernatant was then collected and centrifuged at 120,000 rpm and 4° C for 4 h using a Beckman Coulter (USA) ultracentrifuge. The samples were then stored in a -80° C freezer until the analysis (Gu et al., 2012; Li et al., 2016).

Table 1. CMT scores and cell count results for collected milk samples from HF cows
Çizelge 1. SA ineklerinden toplanan süt numuneleri için CMT skorları ve hücre sayısı sonuçları

Sample No *Örnek No*	CMT score *CMT skoru*	Cell count *Hücre sayısı*	Clinical Signs *Klinik bulgular*	Group *Grup*
1	2+	≥200.000	None	subclinical mastitis
2	3+	≥200.000	None	subclinical mastitis
3	2+	≥200.000	None	subclinical mastitis
4	3+	≥200.000	None	subclinical mastitis
5	-	≤200.000	None	normal
6	3+	≥200.000	None	subclinical mastitis
7	-	≤200.000	None	normal
8	2+	≥200.000	None	subclinical mastitis
9	-	≤200.000	None	normal
10	2+	≥200.000	None	subclinical mastitis
11	-	≤200.000	None	normal
12	2+	≥200.000	None	subclinical mastitis
13	2+	≥200.000	None	subclinical mastitis
14	2+	≥200.000	None	subclinical mastitis
15	3+	≥200.000	None	subclinical mastitis
16	2+	≥200.000	None	subclinical mastitis
17	3+	≥200.000	None	subclinical mastitis
18	3+	≥200.000	None	subclinical mastitis
19	-	≤200.000	None	normal
20	3+	≥200.000	None	subclinical mastitis
21	2+	≥200.000	None	subclinical mastitis
22	2+	≥200.000	None	subclinical mastitis
23	3+	≥200.000	None	subclinical mastitis
24	3+	≥200.000	None	subclinical mastitis
25	2+	≥200.000	None	subclinical mastitis
26	-	≤200.000	None	normal
27	3+	≥200.000	None	subclinical mastitis
28	2+	≥200.000	None	subclinical mastitis
29	3+	≥200.000	None	subclinical mastitis
30	3+	≥200.000	None	subclinical mastitis
31	3+	≥200.000	None	subclinical mastitis
32	2+	≥200.000	None	subclinical mastitis
33	2+	≥200.000	None	subclinical mastitis
34	3+	≥200.000	None	subclinical mastitis
35	2+	≥200.000	None	subclinical mastitis
36	3+	≥200.000	None	subclinical mastitis
37	2+	≥200.000	None	subclinical mastitis
38	3+	≥200.000	None	subclinical mastitis
39	2+	≥200.000	None	subclinical mastitis
40	3+	≥200.000	None	subclinical mastitis

Total RNA Isolation and cDNA Synthesis

Total RNA was isolated from the obtained exosomes samples using Trizol (Invitrogen, USA). RNA concentration was evaluated with NanoDrop (Epoch Microplate Spectrophotometer, USA). RNA quality was determined using gel electrophoresis (Thermo Fisher). cDNA synthesis was conducted using the miScript Reverse Transcription Kit (Qiagen, Germany). cDNA samples were stored at -20 ^{0}C for the further analysis (Ozdemir and Comakli, 2018).

Real time PCR

The expression levels of miR-21, miR-146a, miR-155, miR-222, miR-383, miR-200a, miR-205, miR-122, and miR-182 were determined using RT-PCR (BioRad CFX96). QuantiTect SYBR® Green PCR Kits (Qiagen, Germany) were used in this experiment. The CT/CQ values were evaluated with the $2^{-(CTmiRNA-CT5SRNA)}$ method (Ozdemir and Comakli, 2018). 5S snRNA was used as internal control. The primers for miRNAs were designed using Primer 3 program. The primers are shown in Table 3.

Table 2. CMT scores and cell count results for collected milk samples from DAK cows
Çizelge 2. DAK ineklerinden toplanan süt numuneleri için CMT skorları ve hücre sayısı sonuçları

Sample No *Örnek no*	CMT score *CMT skoru*	Cell count *Hücre sayısı*	Clinical Signs *Klinik bulgular*	Group *Grup*
1		≤200.000	None	normal
2	2+	≥200.000	None	subclinical mastitis
3		≤200.000	None	normal
4	3+	≥200.000	None	subclinical mastitis
5	2+	≥200.000	None	subclinical mastitis
6	2+	≥200.000	None	subclinical mastitis
7	2+	≥200.000	None	subclinical mastitis
8	2+	≥200.000	None	subclinical mastitis
9	3+	≥200.000	None	subclinical mastitis
10	2+	≥200.000	None	subclinical mastitis
11	2+	≥200.000	None	subclinical mastitis
12		≤200.000	None	normal
13		≤200.000	None	normal
14	2+	≥200.000	None	subclinical mastitis
15	3+	≥200.000	None	subclinical mastitis
16		≤200.000	None	normal
17	3+	≥200.000	None	subclinical mastitis
18	3+	≥200.000	None	subclinical mastitis
19	2+	≥200.000	None	subclinical mastitis
20	2+	≥200.000	None	subclinical mastitis
21		≤200.000	None	normal
22	2+	≥200.000	None	subclinical mastitis
23	2+	≥200.000	None	subclinical mastitis
24	2+	≥200.000	None	subclinical mastitis
25		≤200.000	None	normal
26	2+	≥200.000	None	subclinical mastitis
27	3+	≥200.000	None	subclinical mastitis
28	3+	≥200.000	None	subclinical mastitis
29	2+	≥200.000	None	subclinical mastitis
30	2+	≥200.000	None	subclinical mastitis
31	3+	≥200.000	None	subclinical mastitis
32	2+	≥200.000	None	subclinical mastitis
33	3+	≥200.000	None	subclinical mastitis
34	2+	≥200.000	None	subclinical mastitis
35	3+	≥200.000	None	subclinical mastitis
36	2+	≥200.000	None	subclinical mastitis
37	3+	≥200.000	None	subclinical mastitis
38	2+	≥200.000	None	subclinical mastitis
39	3+	≥200.000	None	subclinical mastitis
40	3+	≥200.000	None	subclinical mastitis

Table 3. Summary of miRNA primers sequences for the RT-PCR
Çizelge 3. RT-PCR için miRNA primer dizileri

Name (İsim)	*Sequence (5′ → 3′) (Dizi)*	*Length (nt) (Uzunluk)*	*GC (%)*
bta-miR-21	*TAGCTTATCAGACTGATGTTGACT*	*24*	*40.9*
bta-miR-146a	*CCCATGTGTATCCTCAGCTTT*	*21*	*59.1*
bta-miR-155	*TGTTAATGCTAATCGTGATTT*	*21*	*77.3*
bta-miR-222	*AGCTACATCTGGCTACTGGGT*	*21*	*45.5*
bta-miR-383	*AGATCAGAAGGTGATTGTGGCT*	*22*	*52.4*
bta-miR-200a	*TAACACTGTCTGGTAACGATGTT*	*23*	*39.1*
bta-miR-205	*TCCTTCATTCCACCGGAGTCTG*	*22*	*54.54*
bta-miR-122	*TGGAGTGTGACAATGGTGTTTG*	*22*	*45.45*
bta-miR-182	*TTTGGCAATGGTAGAACTCACACT*	*24*	*41.6*

Statistical analysis

One-Way Analysis of Variance (ANOVA, IBM SPSS 20) used to detect statistically differences miRNA expressions between normal and *M. Bovis* positive milk samples. Statistically differences were considered to be significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$ (Ozdemir and Comakli, 2018).

Permission for the study was obtained from the Animal Experiments Local Ethics Committee of Ataturk University with the decision of the meeting dated 28.07.2017 and numbered 1700212429

Table 4. Pathogens detection results in the HF milk samples
Çizelge 4. SA ırkına ait süt örneklerinde patojenlerin saptanma sonuçları

Sample No (*Örnek No*)	Target result (*Hedef sonuç*)	Ct value (*Ct deperi*)	Pathogen type (*Patojen tipi*)
1	positive	20	*Streptococcus agalactiae*
2	positive	22	*Staphylococcus aureus*
3	positive	21	*Mycoplasma bovis*
4	positive	20	*Mycoplasma spp*
5	negative	ND	-
6	positive	22	*Streptococcus agalactiae*
7	negative	ND	-
8	positive	23	*Streptococcus agalactiae*
9	negaitve	ND	-
10	positive	25	*Staphylococcus aureus*
11	negative	ND	-
12	positive	24	*Mycoplasma bovis*
13	positive	20	*Mycoplasma bovis*
14	positive	22	*Mycoplasma bovis*
15	positive	21	*Streptococcus agalactiae*
16	positive	23	*Streptococcus agalactiae*
17	positive	24	*Streptococcus agalactiae*
18	positive	25	*Mycoplasma bovis*
19	negative	ND	-
20	positive	22	*Streptococcus agalactiae*
21	positive	23	*Streptococcus agalactiae*
22	positive	24	*Staphylococcus aureus*
23	positive	23	*Mycoplasma spp*
24	positive	20	*Staphylococcus aureus*
25	positive	20	*Mycoplasma bovis*
26	negative	ND	-
27	positive	20	*Mycoplasma bovis*
28	positive	20	*Streptococcus agalactiae*
29	positive	22	*Mycoplasma bovis*
30	positive	20	*Mycoplasma bovis*
31	positive	24	*Mix infection*
32	positive	26	*Mix infection*
33	positive	22	*Mix infection*
34	positive	19	*Mix infection*
35	positive	25	*Mix infection*
36	positive	21	*Mix infection*
37	positive	27	*Mix infection*
38	positive	23	*Mix infection*
39	positive	22	*Mix infection*
40	positive	20	*Mix infection*

RESULTS

Detection of pathogens in milk samples

All milk samples were analyzed for common pathogens associated with mastitis. In the HF milk samples, *S. agalactiae* (n = 9), *S. aureus* (n = 4), *M. bovis* (n = 9), other *Mycoplasma spp.* (n = 2), and mix infected (n=10) were detected (Table 4). In the DAK milk samples, *S. agalactiae* (n = 8), *S. aureus* (n = 8), *M. bovis* (n = 6), other *Mycoplasma spp.* (n = 1), and mix infected (n=10) were detected (Table 5).

Table 5. Pathogens detection results in the DAK milk samples
Çizelge 5. DAK ırkına ait süt örneklerinde patojenlerin saptanma sonuçları

Sample No (*Örnek No*)	Target result (*Hedef Sonuç*)	Ct value (*Ct değeri*)	Pathogen type (*Patojen tipi*)
1	negative	ND	-
2	positive	23	*Staphylococcus aureus*
3	negative	ND	-
4	positive	19	*Staphylococcus aureus*
5	positive	23	*Staphylococcus aureus*
6	positive	25	*Streptococcus agalactiae*
7	positive	22	*Mycoplasma bovis*
8	positive	21	*Streptococcus agalactiae*
9	positive	22	*Mycoplasma bovis*
10	positive	21	*Staphylococcus aureus*
11	positive	22	*Mycoplasma bovis*
12	negative	ND	-
13	negative	ND	-
14	positive	22	*Mycoplasma bovis*
15	positive	24	*Streptococcus agalactiae*
16	negative	ND	-
17	positive	20	*Streptococcus agalactiae*
18	positive	20	*Streptococcus agalactiae*
19	positive	23	*Staphylococcus aureus*
20	positive	21	*Streptococcus agalactiae*
21	negative	ND	-
22	positive	24	*Staphylococcus aureus*
23	positive	21	*Mycoplasma spp*
24	positive	22	*Staphylococcus aureus*
25	negative	ND	-
26	positive	22	*Mycoplasma bovis*
27	positive	23	*Mycoplasma bovis*
28	positive	25	*Streptococcus agalactiae*
29	positive	19	*Streptococcus agalactiae*
30	positive	21	*Staphylococcus aureus*
31	positive	20	*Mix infection*
32	positive	21	*Mix infection*
33	positive	25	*Mix infection*
34	positive	22	*Mix infection*
35	positive	22	*Mix infection*
36	positive	23	*Mix infection*
37	positive	21	*Mix infection*
38	positive	19	*Mix infection*
39	positive	24	*Mix infection*
40	positive	22	*Mix infection*

Detection of M. bovis uvrC gene using the RT-PCR method

RT-PCR was performed to detect *M. bovis uvrC* gene in milk samples which were identified as normal and subclinical mastitis. The milk samples identified as subclinic mastitis were found to be *M. bovis* positive and the normal milk samples were found to be *M. Bovis* negative (Table 6).

Relative expression profiles of miRNA candidate biomarkers

The expression levels of inflammation-related miRNAs in exosomes from the mastitis milk samples and normal milk samples from HF (n=9) and DAK (n=6) cows were analyzed. The results showed that the expression levels of miR-21, miR-146a, miR-155, miR-222, miR-383, miR-200a, miR-205, miR-122, and miR-182 were markedly upregulated in the mastitis milk samples as compared with that in the normal milk samples ($p < 0.05$). Among the miRNA candidate biomarkers, miR-21 and miR-222 ($p < 0.01$) were significantly upregulated in mastitis milk from HF cows, and miR-146a and miR-383 were significantly upregulated in mastitis milk from DAK cows ($p < 0.01$) (Figs. 1, 2, 3).

Table 6. Detection of *M. bovis uvrC* in serial 10-fold dilutions by RT-PCR
Çizelge 6. RT-PCR ile seri 10 kat seyreltmelerde M. bovis uvrC saptanması.

Dilutions	10^0	10^1	10^2	10^3	10^4
Real-time PCR[a]	Positive (3/3)	Positive (3/3)	Positive (3/3)	Positive (3/3)	Positive (3/3)
Ct values[b]	21.2 ± 0.2	24.2 ± 0.1	28.9 ± 0.4	33.5 ± 0.6	37.9 ± 0.1

a results from 5 analyses. b mean values and standard errors from 3 measurements
a 5 analizden elde edilen sonuçlar. b 3 ölçümdeki ortalama değerler ve standart hatalar

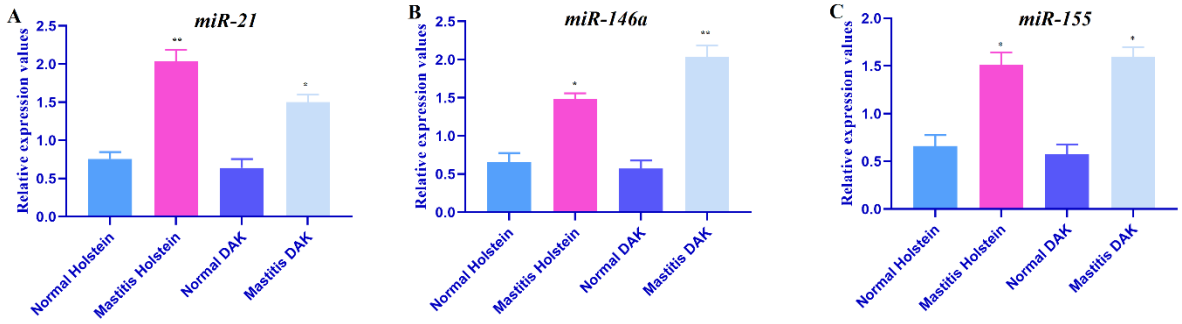

Figure 1. Relative expression profiles of miR-21, miR-146a, and miR-155 in the normal and mastitic milk of HF and DAK
Şekil 1. SA ve DAK ırkına ait mastitli ve normal sütlerdeki miR-21, miR-146a ve miR-155'in ekspresyon profili.

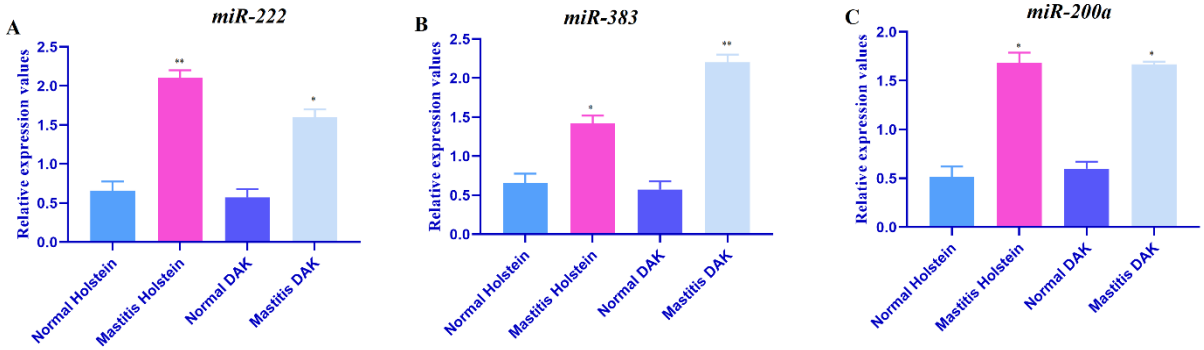

Figure 2. Relative expression profiles of miR-222, miR-383, and miR-200a in the normal and mastitic milk of HF and DAK.
Şekil 2. SA ve DAK ırkına ait mastitli ve normal sütlerdeki miR-222, miR-383 ve miR-200a'nın ekspresyon profili.

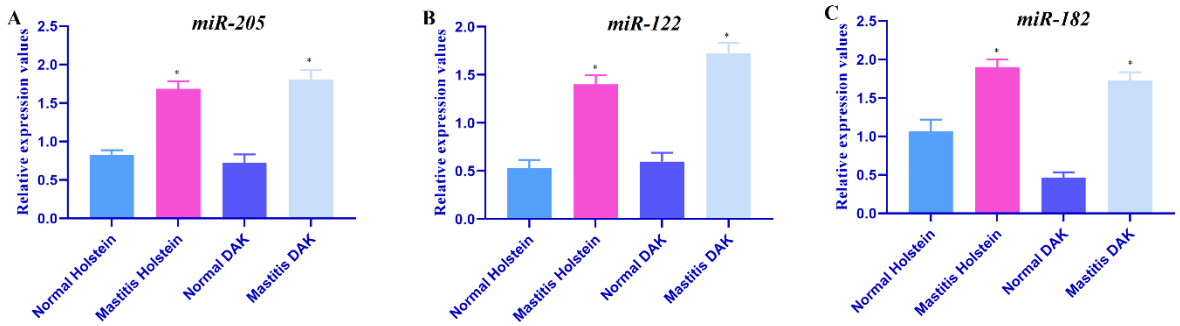

Figure 3. Relative expression profiles of miR-205, miR-122, and miR-182 in the normal and mastitic milk of HF and DAK.
Şekil 3. SA ve DAK ırkına ait mastitli ve normal sütlerdeki miR-205, miR-122 ve miR-182'nin ekspresyon profili.

DISCUSSION

Failure to detect subclinical mastitis promptly allows the infection to spread rapidly (Halasa et al., 2007; Hughes and Watson, 2018). Increasing evidence in recent years suggests that *M. bovis* is a major cause of severe mastitis infections (Vahanikkila et al., 2019; Murai and Higuchi, 2019; Josi et al., 2018) and that *M. bovis* is more frequently detected than other pathogens in subclinical mastitis (Al-Farha et al., 2017; Fox 2012; Nicholas et al., 2016). Therefore, the development of

diagnostic methods that can detect subclinical mastitis at an early stage is important. In our study, *M. bovis* was detected in 9 of 40 milk samples from HF cows and in 6 of 40 milk samples from DAK cows. The results of the CMT and cell counting methods confirmed showed that the animals had subclinical mastitis.

Previous research demonstrated that changes in miRNA expression levels played an important role in inflammatory infections, such as mastitis. Recent studies reported that miRNA expression levels in cows with mastitis were altered in mammary epithelial cells, milk exosomes, and mammary gland tissue (Sheedy and O'Neill, 2008; Jin et al., 2014; Naeem et al., 2012; Lawless et al., 2013; Sun et al., 2015; Li et al., 2015). In the current study, the expression levels of various miRNAs were upregulated in milk infected with *M. bovis*, suggesting that miRNAs may play a role in bovine mastitis caused by *M. bovis*. The findings of this study point to the potential value of these molecular-based biomarkers of mastitis in milk infected with *M. bovis*.

HF cattle are bred in Aegean Turkey, Marmara, and the Mediterranean region, and are known to have the highest milk yield in the world; however, the HF is a sensitive breed against epidemics and parasitic diseases, climatic conditions, and unfavorable stable conditions despite the high milk yield (Akyüz, 2008). DAK is a breed bred in high-altitude areas, especially in Erzurum located in the Eastern Anatolia Region of our country, and is resistant to harsh winters and inappropriate barn conditions, inadequate care and feeding, and epidemics and parasitic diseases (Özdemir, 2011). miRNA candidate biomarkers, including miR-21 and miR-222 were significantly upregulated in mastitis milk from HF cows, whereas miR-146a and miR-383 were significantly upregulated in mastitis milk from DAK cows. This was the important point of this study. Particularly, miR-146a and miR-383 have more gene targets that are related with inflammatory pathways compared to other miRNA candidates. Furthermore, miR-21 and miR-222 have gene targets that are related to both inflammatory pathways and milk synthesis. These results may reveal the phenotypic differences between the two races.

Liquid isolated from body fluids, such as blood, milk and urine, facilitates the diagnosis of diseases (Weber et al., 2010). Previous research indicated that circulating miRNAs in blood, milk, saliva, and urine could be used as diagnostic or prognostic markers in various diseases (Larrea et al., 2016). The suitability of miRNAs in bovine milk as biomarkers for mastitis caused by *M. bovis* was evaluated in the present study. The results indicated that many miRNAs had high predictive values, with high sensitivity and specificity in terms of *M. bovis*-positive versus negative milk. The results illustrate the potential of miRNAs in milk as biomarkers of mastitis.

In dairy cows, mastitis cases could occurred as a mix infection. In this study, a total of 20 mix infections were found for both HF and DAK cows, which was considered to be quite high for particular study. The spread of mixed infections is faster and more difficult to treat. Considering all these, more effective methods should be developed for early diagnosis and treatment of mastitis cases.

CONCLUSION

In conclusion, the expression levels of miR-21, miR-146a, miR-155, miR-222, miR-383, miR-200a, miR-205, miR-122, and miR-182 were significantly upregulated in *M. bovis*-positive milk from HF and DAK cows. Our findings suggest that inflammation-related miRNA expression levels in HF and DAK cow milks was altered in the presence of mastitis and that these miRNAs could be used as biomarkers of bovine mastitis caused by *M. bovis*.

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Statement of Conflict of Interest

Authors have declared no conflict of interest.

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