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1 **Milk microRNA-146a as a potential biomarker in bovine tuberculosis**

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26 **Summary**

27 In this research communication we exploited the potential use of milk microRNAs (miRs) as
28 biomarker for bovine tuberculosis (bTB). bTB is a zoonotic disease caused by *Mycobacterium bovis*
29 which affects animal health influencing herd economic sustainability. Diagnosis is based on skin
30 delayed-type hypersensitivity reaction and quantification of interferon gamma but both techniques
31 are influenced by several confounding factors. Thus, new methods for early diagnosis are required
32 and miRs have been used as promising biomarker for both infectious and non-infectious diseases.
33 For doing that, we analyzed the expression of four immune-related miRs in 200 cows grouped in
34 cases and controls respect to positivity to tuberculosis. The analysis showed a different magnitude
35 expression in both groups indicating that active tuberculosis could influence miRs expression. Then,
36 we used expression values of miR-146a, the highest differentially expressed miR, for ROC analysis.
37 In order to determine a test cut-off value for miR-146a expression that would differentiate cases and
38 controls, a value for the miR-146a expression higher than 8 was selected as this gave a test
39 specificity and sensitivity of 80.0% and 86.0% respectively. These values confirm the possibility of
40 using miR-146a as milk prognostic biomarker for bovine tuberculosis.

41

42

43 **Keywords:** microRNAs, biomarker, tuberculosis, case-control study, dairy cows

44

45 Bovine tuberculosis (bTB) is a zoonotic disease caused by *Mycobacterium bovis*, a bacteria
46 belonging to the *Mycobacterium tuberculosis* complex. Disease development is influenced by host
47 genetic background (Papaianni *et al*, 2017) and by a proper immune response. bTB significantly
48 affects animal health influencing herd economic sustainability due to reduction in productivity in
49 severely affected animals. Thus, control and eradication of disease is principally based on removal
50 and slaughter of infected animals following early diagnosis of disease (Vordermeier *et al*, 2016).
51 Diagnosis is based on skin test where the intradermal injection of crude preparation from bacteria
52 gives a delayed-type hypersensitivity reaction in positive animals. This technique is often associated
53 with quantification of interferon gamma in ex-vivo stimulated cell of tested animals. However,
54 these methods are influenced by several confounding factors (Pai *et al*, 2004) and therefore new
55 potential biomarkers are required. microRNAs (miRs) are small non-coding RNA that influence
56 gene expression controlling mRNA degradation (Bartel, 2004). Differential expression of miRs is
57 been often associated with non-infectious (Jeffrey S.S., 2008) and infectious diseases like TB (Vegh
58 *et al*, 2013). Moreover, recent studies have shown that extracellular miRs are stably recovered from
59 body fluid including milk (Weber, 2010) where they have been associated with various biological
60 processes, including immune response (Kosaka *et al*, 2010). Based on these studies, we have
61 measured the expression of some miRs from milk of active infected cows compare with healthy
62 ones. Finally, we have evaluated the potential use of miR-146a as prognostic biomarker for
63 diagnosis of tuberculosis.

64

65 **Materials and methods**

66 **Samples collection**

67 Animals included in the study were from 3 different herds declared infected and were enrolled
68 already in our previous study. As described, they were grouped in cases and controls according to
69 reactivity to resuscitation factor B (Capparelli *et al*, 2013). Milk samples were stored at 4°C and
70 immediately processed once arrived to the laboratory. Then, samples were centrifuged at $3,000 \times g$
71 for 15 min at room temperature. The supernatant was recovered and further centrifuged at $15,000 \times$
72 g for 15 min at 4°C. The milk whey was recovered and stored at -80°C for RNA extraction.

73

74 **RNA Extraction**

75 Before proceeding with RNA extraction from whey milk, 5 fmol of exogenous cel-miR-39 were
76 spiked-in. RNA was isolated using Trizol LS (Thermofisher) following manufacturer's instruction
77 and quantified using NanoDrop. Only samples with both 260/280 and 260/230 ratios >1.8 were
78 used for further investigation.

79

80 **Real-Time Reverse Transcriptase Quantitative PCR**

81 One microgram of total RNA was reverse transcribed using TaqMan™ MicroRNA Reverse
82 Transcription Kit (Thermo FisherScientific) according to the manufacturer's protocol. Quantitative
83 PCR was performed using a TaqMan Fast Advanced Master Mix kit and a StepOne Plus Real-Time
84 PCR system (Thermo Fisher Scientific). The TaqMan MicroRNA Assays used in this study and
85 their Taqman assay IDs are as follows: bta-miR-146a (ID: 005896), bta-miR-29c (ID: 000587), bta-
86 miR-155 (ID: 002623) and bta-miR99b (ID: 000436) . Thermal cycling was conducted according to
87 the manufacturer's recommended program and using cel-miR-39 as internal control for relative
88 quantification.

89

90 **Statistical Analysis**

91 All of the milk samples were taken from Holstein-Friesian cows. To reduce potential confounders,
92 all subjects were lactating cow between 40 and 90 month of age and a screening for mastitis was
93 performed immediately in the field using a California mastitis test with a commercial tester for
94 exclude mastitis coinfection. To curb quarter bias, quarter samples were mixed. Data analysis and
95 Receiving operating curve (ROC) were performed using GraphPad Prism 6 (GraphPad Software
96 Inc., San Diego, CA). Data were analyzed using a parametric unpaired t test, and $P < 0.05$ was
97 considered statistically significant.
98

99 **Results and discussion**

100 We compared miRs expression in 200 milk samples of Holstein-Friesian cows. Samples were
101 previously grouped in cases (100 animals actively infected) and controls (100 animals negative)
102 according to our previous study (Capparelli *et al*, 2013). In detail, bta-miR-146a, bta-miR-29, bta-
103 miR-155, bta-miR99b were chosen according with previous study where was shown they were
104 differentially regulated in bovine during infection with *M.bovis* [6]. Using a qRT-PCR, we analyzed
105 the expression of the above miRs and all of them were significantly up-regulated in cases versus
106 controls (Table 1). In detail, miR-146a shows the highest difference between cases and controls and
107 the expression data were subjected to ROC analysis to determine if miR-146a could serve as a
108 sensitive and specific prognostic marker identifying animals with active TB. This analysis show that
109 miR-146a could discriminate significantly between cases and controls animals (area under the
110 curve: 0.795 [95% CI: 0.7290 to 0.8614, $p<0.001$] (Figure 1). In order to determine a test cut-off
111 value for miR-146a expression that would differentiate cases and controls, a value for the miR-146a
112 expression higher than 8 was selected as this gave a test specificity of 80.0% (i.e. 20/100 detection
113 of false positive miR-146s responses in control animals). Using this cut-off,miR-146a positive
114 responses were identified in 16/100 controls, giving a test sensitivity of 86.0%. The prognostic
115 accuracy of miR-146a to distinguish cases from controls was also highlighted by calculating the
116 odds ratio after applying the cut-off value described above. Thus, the diagnostic odds ratio was
117 established as being 24, indicating that for an animal showing significant induction of miR-146a in
118 milk the probability of it developing tuberculosis is 24-fold higher than not having it.

119

120 **Conclusion**

121 This is the first study to demonstrate that miRs, named bta-miR-146a, bta-miR-29, bta-miR-155 and
122 bta-miR99b are differentially regulated in milk from tuberculosis affected dairy cows. This result
123 shows the potential of miRNA in milk for use as a biomarker for bovine tuberculosis. In particular,

124 we have shown that miR-146a can be used as potential biomarker candidate active tuberculosis
125 infection in cows with 80.0% and 86.0% of specificity and sensitivity respectively.

126

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130 agro-alimentari”)

131

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154

155

	Cases (100)	Control(100)	P-value ^a
bta-miR-146a	9.19 ± 2.83	6.81 ± 1.76	<0.001
bta-miR-29c	6.78 ± 1.22	5.24 ± 1.67	<0.001
bta-miR-155	4.25 ± 1.22	3.44 ± 1.01	<0.001
bta-miR-99b	5.74 ± 1.32	4.56 ± 0.87	<0.001

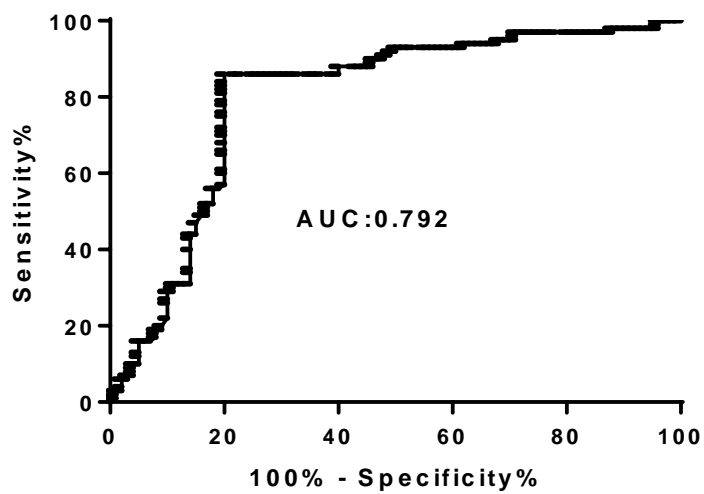
156

157 **Table 1.** Relative microRNAs expression in milk from both active TB (cases) and
158 control (negative) cows.

159 ^a Student's t-test.

160

161



162

163 **Figure 1.** miR-146a as a biomarker of active tuberculosis in milk. Receiver operating characteristic
164 (ROC) analysis to assess efficacy of miR-146a as a biomarker of tuberculosis. AUC, area under the
165 curve.

166